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BIODIESEL FROM MICROALGAE: THE LINK BETWEEN PHOTOSYNTHESIS AND PRODUCTIVITY IN *NANNOCHLOROPSIS GADITANA*

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A chi ho nel cuore

A todos aquellos que llevo en mi corazon

Pour tous ceux qui sont dans mon coeur

To everyone in my heart

ABBREVIATIONS

Chl, Chlorophyll

Car, Carotenoids

PSI, Photosystem I

PSII, Photosystem II

LHC, Light Harvesting Complex

Cyt b_6f, Cytochrome b_6f complex

ECS, Electrochromic Shift

DCMU, - 3-(3,4-dichlorophenyl)-1,1-dimethylurea

HA, hydroxylamine

DBMIB, Dibromothymoquinone

CEF, Cyclic Electron Flow

LEF, Linear Electron Flow

Fv/Fm, Photosystem II Quantum Yeld

qP, Photochemical quenching

NPQ, Non Photochemical Quenching

OD, Optical Density

ROS, Reactive Oxygen Species

N, nitrogen; N+, nitrogen excess: N-, nitrogen deprivation

VDE, Violaxanthin De-Epoxidase

SUMMARY

Most biodiesel is currently produced from oleaginous crops like sunflower or soybean. However, the use of crop plants for this kind of applications presents many limitations. One is that even in the most producing species oils are less than 5% of total biomass basis, thus limiting the possible productivity.

On the contrary, in some species of microalgae more that 50% of the total dry matter is represented by lipids. Algae have thus a promising potential as feedstock for biodiesel production. However, a strong research effort is still needed to fulfill their potential in large scale production to at least partially replace fossil fuels. Species belonging to the genus *Nannochloropsis* are considered among the most promising for biodiesel production because of their ability to accumulate large amounts of lipids.

The general aim of this work was to analyze the role of photochemical efficiency in the productivity of the marine microalga *Nannochloropsis gaditana*. The efficiency in using light is in fact fundamental to have productive large scale algae cultivation.

As for all photosynthetic organisms, light is a major factor influencing algae growth since it provides all the energy supporting metabolism. However, radiation can also be dangerous because, if in excess, it may drive the formation of reactive oxygen species (ROS) and oxidative stress affecting algae productivity.

In the second chapter the effect of different light regimes on biomass and lipids productivity of *Nannochloropsis gaditana* is assessed. Results showed that this organism is able to adapt to a wide range of light intensities by activating an acclimative response and maintain growth rate. The exposition to different light intensities did not show differences in lipids accumulation.

In the third chapter similar experiments were performed in the presence of excess CO_2, one major factor for algae large scale cultivation. Algae are in fact able to exploit CO_2 externally provided to support and increase the biomass productivity. Here, the combined effect of light intensity and carbon dioxide on *Nannochloropsis gaditana* growth and lipids productivity was analyzed. Results showed that excess CO_2 strongly increased the growth rate at all light intensities and that under strong illumination lipids synthesis was stimulated. Thus, strong illumination does not induce lipids accumulation per se but it contributes when excess CO_2 is also present.

A major signal inducing lipids accumulation in algae is nutrients limitation and nitrogen starvation in particular. In these conditions, *Nannochloropsis* cells can accumulate up to 70% of dry weight as lipids. In the fourth chapter, the influence of this macronutrient availability on photosynthetic efficiency in *Nannochloropsis* cells was assessed. Results showed that nitrogen depletion affects the whole photosynthetic chain with Photosystem II (PSII) particularly affected. As a result linear electron flow from PSII to PSI is strongly decreased. *Nannochloropsis* cells were still able to maintain a residual photosynthetic efficiency exploiting alternative electron flow such as cyclic electron transport around PSI.

In conclusion this work showed that *Nannochloropsis* could be considered a good candidate for the outdoor biodiesel production because of its good growth rate, its lipids accumulation and its ability to adapt to very different illumination intensities that usually characterize the natural environment. However, strong nutrient stress treatments, although effective in inducing large relative lipids content, limit the overall process productivity because of a reduced photosynthetic efficiency and are thus not suitable in a perspective of a large scale production. Finally, the observation that cells are able to adapt their photosynthetic apparatus to respond to this type of stress opens the possibility of finding optimized conditions where a nutrient limitation can stimulate the lipids accumulation without affecting photosynthetic efficiency or improve responses to nutrient depletion by genetic engineering *Nannochloropsis* cells.

In the fifth chapter nitrogen depletion effects on photosynthetic apparatus were compared with that in the model green alga *Chlamydomonas reinhardtii*, showing even in this case the alteration of the balance between the two photosystems in response to N availability. PSII was confirmed as the major target under N deprivation with the linear electron flow from PSII to PSI showing a decreased amount. However, under nitrogen deprivation the cyclic electron flow was strongly exploited. Concluding, the re-organization of the entire photosynthetic apparatus and the improvement of alternative electron flows instead of LEF in both *Nannochloropsis* and *Chlamydomonas* seem to be a common response to nitrogen deficiency in algae even not strictly correlated from an evolutive point of view.

In appendix 1 the influence of different light intensities was tested using alternation of light and dark cycles with different frequencies, which mimic illumination variations in

a photobioreactor due to mixing. Results showed that *Nannochloropsis* is able to use efficiently very intense light in presence of the optimal frequency of light-dark cycles. In fact, when the frequency was not optimal *Nannochloropsis* suffered a reduced photosynthetic productivity and remarkable photodamages. Thus, the mixing optimization has to be considered a seminal parameter for algae to exploit light energy with the highest efficiency.

In order to understand as much as possible the basic mechanisms for light adaptation, a part of this work was then focused on the biochemical characterization of a protein involved in the regulation mechanism of photosynthesis, the Violaxanthin De-Epoxidase (VDE). In particular, it participates to the xanthophylls cycle, a well known photoprotection mechanism and it is known that at neutral pH, VDE is soluble in the lumen but it binds to the thylakoids upon a conformational change when the pH decreases. In appendix 2, for convenience the VDE belonging to the model higher plant *Arabidopsis thaliana* was studied. In any case, recently, a homologue gene for this protein was found in *Nannochloropsis*. The pH-dependent activation mechanism of this enzyme was analyzed and thanks to an in silico analysis we were able to identify the residues which are putatively involved in the pH dependent conformational change. We also mutated these residues to verify experimentally if they were indeed important for the enzyme activity and results indicated that each identified key residue was sufficient to induce the first step of the activation and all the residues together produced a cooperative effect on the final activity.

RIASSUNTO

Attualmente il biodiesel è prodotto da piante superiori quali girasole e soia definite oleaginose per la loro capacità di accumulare lipidi nei semi. Tuttavia, il loro impiego per la produzione di oli combustibili presenta numerose limitazioni. Ad esempio, nel caso delle specie di piante maggiormente produttive meno del 5% sulla biomassa totale è rappresentato da lipidi.

In alcune specie di microalghe, invece, più del 50% del peso secco è rappresentato da lipidi rendendo tali organismi decisamente competitivi nella possibile produzione di biodiesel. Tuttavia, è necessario ampliare le ricerche nel campo in modo tale da riuscire a sfruttare appieno il potenziale insito nelle alghe per una produzione su larga scala. In questo senso, le specie appartenenti al genere *Nannochloropsis* riscuotono un grande successo, dovuto principalmente alle caratteristiche fondamentali che caratterizzano questo genere, ovvero una buona velocità di crescita accompagnata da una buona produzione in termini di lipidi.

Questo lavoro si propone di analizzare il ruolo svolto dalla fotosintesi nella produttività finale di una microalga marina, *Nannochloropsis gaditana*. L'efficienza nell'utilizzo della luce è infatti un punto cardine per massimizzare la produttività algale su larga scala.

In tutti gli organismi fotosintetici la luce è uno dei fattori che maggiormente ne influenza la crescita dal momento che è essenziale per tutti i processi metabolici. Tuttavia, la radiazione luminosa può in alcuni casi essere fonte di stress. Per esempio, quando la luce incidente sull'organismo è in eccesso può portare alla formazione di forme reattive dell'ossigeno e a stress ossidativi che in qualche modo vanno ad agire sulla produttività delle alghe.

Nel secondo capitolo, colture di *Nannochloropsis gaditana* sono state esposte a diverse intensità luminose e per ciascuna di esse sono stati monitorati il tasso di crescita e la produttività in termini di lipidi. I risultati raccolti hanno dimostrato come *Nannochloropsis* sia in grado di adattarsi a tutte le intensità luminose testate attivando una risposta acclimativa che ne ha permesso la crescita nelle diverse condizioni. Tuttavia l'intensità luminosa non sembra avere un effetto particolare sulla produzione finale di lipidi.

Nel terzo capitolo, colture di *Nannochloropsis* sono state esposte alle stesse intensità luminose del capitolo precedente ma in presenza di un flusso di aria miscelato con anidride carbonica al 5% in quanto questo ultimo componente rappresenta uno dei maggiori fattori utilizzati nella produzione di biodiesel su larga scala. Le alghe infatti sono in grado di sfruttare in modo molto efficiente la CO_2, utilizzandola per incrementare il tasso di crescita. In questo capitolo è stato analizzato l'effetto combinato di luce ed eccesso di CO_2 sulla crescita e sulla produttività lipidica di *Nannochloropsis*. I risultati ottenuti hanno dimostrato come l'eccesso di CO_2 stimoli positivamente la crescita algale, aumentando la quantità di biomassa finale e allo stesso modo la sintesi di lipidi, che in eccesso di luce è fortemente incrementata.

La limitata disponibilità di nutrienti è uno dei maggiori fattori che influenza la sintesi di lipidi nelle alghe, in particolare la mancanza di azoto. In queste condizioni, *Nannochloropsis* è in grado di accumulare lipidi fino al 70% rispetto al peso secco. Nel capitolo quattro è stata quindi studiata l'influenza della carenza di azoto in colture di *Nannochloropsis*. I risultati prodotti hanno stabilito come in carenza di azoto si instauri una riorganizzazione dell'intero apparato fotosintetico confermando il PSII come il maggior target di tale stress producendo, inoltre, come conseguenza una diminuzione del trasporto elettronico lineare dal PSII al PSI e un forte aumento del trasporto ciclico intorno al PSI.

Nannochloropsis, dunque, si presenta come candidato ideale per la produzione di biodiesel grazie al veloce tasso di crescita, alla buona produzione lipidica e all'estremo grado di adattamento alle più diverse intensità luminose che normalmente caratterizzano l'ambiente naturale. Tuttavia, l'utilizzo della forte carenza di azoto per la stimolazione dell'accumulo di lipidi non sembra il metodo migliore per la produzione di biodiesel su larga scala in quanto l'efficienza fotosintetica delle alghe ne è fortemente risentita con una riduzione della produttività finale. Ad ogni modo, il fatto che *Nannochloropsis* sia in grado di modulare il proprio apparato fotosintetico in risposta a cambiamenti ambientali implica la possibilità di trovare un equilibrio tra il grado di stress applicato alle alghe e il mantenimento di una buona efficienza fotosintetica nonché di modificare geneticamente le alghe in modo tale da aumentare la sintesi di lipidi finale.

Nel capitolo cinque gli effetti della carenza di azoto sono stati studiati nell'alga verde *Chlamydomonas reinhardtii*, verificando anche in questo caso come la disponibilità di

azoto influenzi soprattutto il PSII e il trasporto ciclico degli elettroni risulti incrementato in carenza di azoto. Comparando le risposte di *Nannochloropsis* e *Chlamydomonas* si può quindi proporre la riorganizzazione dell'intero apparato fotosintetico e lo sfruttamento di flussi alternativi di elettroni rispetto al comune lineare, come una tipica risposta adattiva alla mancanza di azoto nelle alghe.

Nell'appendice 1 l'influenza delle diverse intensità luminose è stata analizzata applicando alle alghe un'alternanza di cicli luce-buio a diverse frequenze che potessero simulare le condizioni di ombreggiamento dovute al mescolamento presenti in un fotobioreattore. I risultati proposti hanno dimostrato come *Nannochloropsis* sia in grado di sfruttare al meglio anche le intensità luminose molto elevate purché la frequenza dei cicli luce-buio sia ottimale. In caso contrario le cellule sono sottoposte ad una drastica riduzione nell'efficienza fotosintetica con conseguente calo della produttività. L'ottimizzazione del mescolamento, dunque, è da considerarsi come un parametro fondamentale per poter sfruttare pienamente la capacità di utilizzo della luce da parte delle alghe.

Per poter capire nel modo più dettagliato possibile i meccanismi di base che permettono agli organismi fotosintetici di adattarsi alle variazioni di intensità luminosa, una parte di questo lavoro è stata incentrata sullo studio di una proteina coinvolta in un ben noto meccanismo di fotoprotezione, il ciclo delle xantofille. L'enzima preso in considerazione è la Violaxantina De-Epossidasi (VDE) che a pH neutri si trova solubile nel lumen tilacoidale per poi essere in grado di legarsi ai tilacoidi una volta che il pH acidifica, assumendo un cambiamento conformazionale. Nell'appendice 2 è trattata la VDE della pianta superiore *Arabidopsis thaliana*, organismo scelto per comodità sperimentale, anche se recentemente un gene omologo che codifica per la VDE è stato trovato anche in *Nannochloropsis*. Il meccanismo pH-dipendente è stato indagato grazie ad analisi *in silico* che hanno permesso di riconoscere i residui aminoacidici coinvolti nel cambiamento conformazionale della proteina. Tali residui sono stati poi mutati per verificarne l'importanza a livello dell'attività enzimatica e i risultati finali hanno indicato come ciascun singolo residuo sia sufficiente ad indurre i primi *steps* di attivazione del meccanismo pH-dipendente e come tutti i residui insieme cooperino producendo un effetto cumulativo sull'attività finale dell'enzima.

CHAPTER 1

GENERAL INTRODUCTION

1.1. BIODIESEL FROM MICROALGAE

The current world economy strongly relies on fossil fuels which are exploited as fuels for transportation, generation of electrical power and the production of plastic materials. Fossil fuels reserves, however, are finite and according to many analysts world reserves are estimated to exhaust in less than 50 years (Rodolfi et al., 2008), even not considering the present growth rate of the world population and the expansion of general economy which will likely further increase their consumption (Hannon et al., 2010). Furthermore, the massive exploitation of fossil fuels in last decades caused the accumulation of carbon dioxide in the atmosphere driving to an increase of greenhouse effect and global warming (Chisti, 2008, Govoni et al., 2008). Many scientists suggested that continued emissions of greenhouse gases deriving from human activities will have drastic impact on the climate.

It is thus clear the necessity of developing alternative and renewable energy sources able to displace petroleum derivatives (Chisti, 2010). Fuels produced from living organisms, called biofuels, are in perspective a valid alternative for fossil fuels because they are renewable, they are made from a biodegradable mass and they are carbon neutral so they not drive to any net CO_2 release in the atmosphere (Gouveia and Oliveira, 2009). The biofuels more commonly considered are biodiesel, biohydrogen, bioethanol and biogas. Among them, biodiesel has interesting perspectives for the new future. Most biodiesel is currently produced from oleaginous crops like sunflower or soybean. However, the use of crop plants for this kind of applications presents many limitations. One is that even in the most producing species oils are less than 5% of total biomass basis, thus limiting the possible productivity (Jeong et al., 2004, Chisti, 2008).

Furthermore the use of crop plants for biofuels production risk to compete with food crops for arable land.

One promising alternative to plants is the possibility of using other photosynthetic organisms, microalgae, which have several potential advantages with respect to crops (Chisti, 2010, Hannon et al., 2010, Chisti and Yan, 2011). In fact some species of microalgae are able to accumulate large amounts of oil within their cells which can be extracted, processed and refined into transportation fuels using the modern technology. In certain species more that 50% of the total dry matter is represented by lipids, an amount far larger than what is found in plants (Rodolfi et al., 2008). Another favorable characteristic of the microalgae is that the entire microalgal biomass is photosynthetically active while in the plants the leaves represent only a part of the biomass. Microalgae production is not seasonal, while plants in temperate climates are not photosynthetically active for the largest fraction of the year, while microalgae can continuously produce biomass. They generally have a fast growth rate and in optimal conditions they duplicate more than once a day (Chisti, 2008). They do not use arable land and potable water and, as a consequence of their higher productivity, they need smaller areas for the cultivation. Microalgae, also, are more efficient in CO_2 fixation with respect to vascular plants and they are responsible for more than 40% of the global carbon fixation, with the majority of this productivity coming from marine microalgae (Morosinotto et al., 2009, Hannon et al., 2010). Plants use only light energy to support growth, while microalgae are mixotrophic organisms and the supply of organic residues may support growth when light is limiting. Finally, algae production strains also have the potential to be bioengineered, allowing improvement of lipids production (Radakowitz et al., 2010) but also of other specific compounds deriving from algae that could be used in other fields. For example algal systems can be used for wastewater

treatment and bioremediation to capture carbon, nitrogen and phosphorus from specialty industrial, municipal and agriculture wastes (Jiang et al., 2011, Sivakumar e al., 2012). Moreover, microalgae-derived products can also be used in the production of cosmetics, food or food additives. In fact, some marine algal species have been optimized for overproduction of products including astaxanthin, β-carotene, omega-3-fatty acid, vitamin E and other pigments (Del Campo et al., 2007, Guedes et al., 2011, Sivakumar e al., 2012).

1.2. *NANNOCHLOROPSIS*: THE ALGA AND THE BIODIESEL PRODUCER

The large potential of algae for the biofuels production is now widely appreciated. However, it is also recognized that research and development efforts aimed at improving microalgal production and lipid yield are required to fulfill this potential and making the large scale production a true reality (Takeshita, 2011). One first fundamental step is to choose a suitable organism among the large variability of algae. The putative candidate for the biodiesel production needs to present some features, the main ones being fast growth rate and high lipid productivity. This is the case of the species belonging to the genus *Nannochloropsis* which present good growth rates (Sforza et al., 2010) and can accumulate large amounts of lipids that can reach concentrations up to 65-70% of total dry weight (Boussiba et al., 1987, Hodgson et al., 1991, Rodolfi et al., 2008). For the majority of studies presented in this work, *Nannochloropsis gaditana* was considered as the model organism (figure 1).

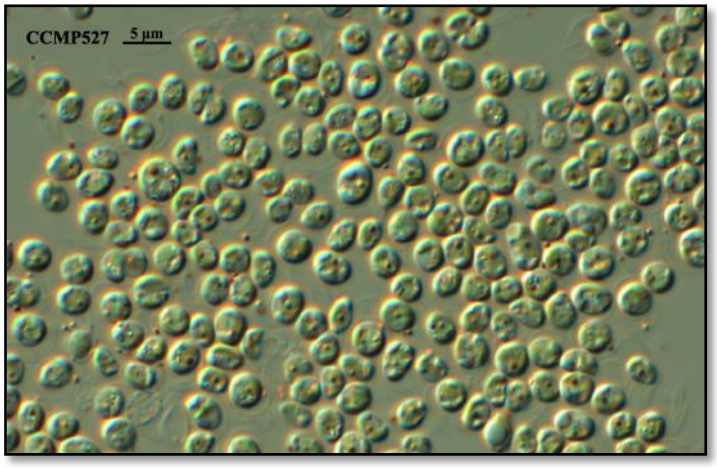

Figure 1. *Nannochloropsis gaditana* Lubiàn (Provasoli-Guillard National Center for Marine Algae and Microbiota, https://ncma.bigelow.org/)

Nannochloropsis gaditana belongs to the infra-reign of Heterokonta (figure 2), usually characterized by organisms with two *flagella* at least during one part of the life cycle (Lee, 2008), although *Nannochloropsis* species appears to have lost this feature. In particular *N. gaditana* belongs to Eustigmathophyceae (Riisberg et al., 2009), an algal class composed mainly by species living in a coastal environment. The name of this class derives from a particular vesicular structure in the cytoplasm defined eye-spot or *eustigma* which normally enables an oriented movement response with respect to the direction and intensity of incident light on the cell in flagellated algae (Kreimer, 2009). As in the case of *flagella*, the *eustigma* in *Nannochloropsis* seems to be not present, at least not in all growing stages. However this aspect is debated (Lubiàn, 1982, Santos et al., 1995).

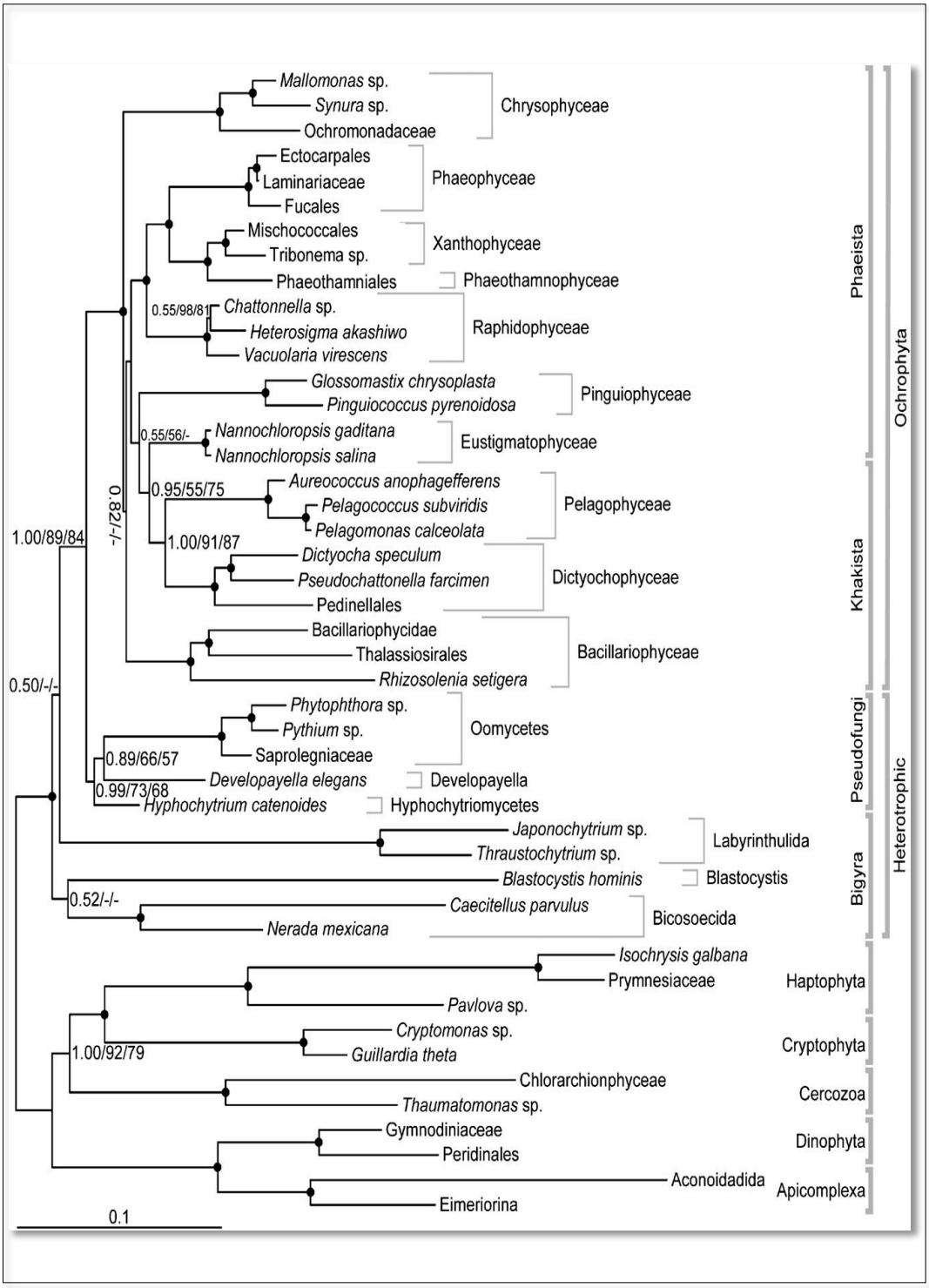

Figure 2. Proteins and rDNA based phylogenetic tree of Heterokonta (Riisberg et al., 2009).

Only one species of the genus belongs to fresh water while the others live in a marine environment, as *N. gaditana*. This species has cells of reduced size (3-5 µm) (Hibberd, 1981) which present a single chloroplast occupying most of the cell volume (Lubiàn, 1982, Lee, 2008) (figure 3). *Nannochloropsis* shows a peculiar pigments content, presenting only chlorophyll (Chl) a and lacking other accessory chlorophylls such as Chl b or c. Violaxanthin and vaucheraxanthin are the most represented carotenoids in the cells but also secondary carotenoids as astaxanthin and cantaxanthin have been detected in peculiar growing conditions (Lubiàn et al., 1986, Lubiàn et al., 2000). In some particular conditions of stress such as nutrient deprivation or high light, *Nannochloropsis* seems to store these secondary carotenoids in a vesicular structure similar to the *eustigma* (Lubiàn, 1982) but with a still unknown function in this species.

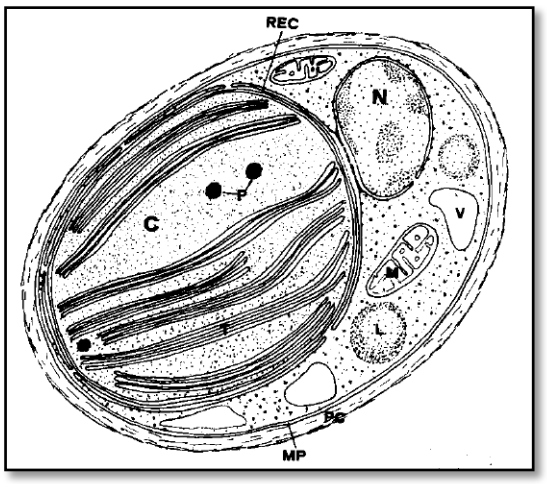

Figure 3. *Nannochloropsis gaditana* Lubiàn ultrastructure (Lubiàn, 1982). REC endoplasmic reticulum T thylakoids V vacuole N nucleus M mitochondrion L lipids droplet PC cell wall MP plasma membrane C chloroplast

Nannochloropsis grows autotrophically at high pH value around 7.5-8 (Sukenik et al., 2009) and salinities from 22 to 49 g/L even if the best condition for this parameter was assed at 31 g/L at 22 °C (Hu and Gao, 2006). However, it is able to utilize glucose, ethanol and glycerol for mixotrophic and heterotrophic growth (Fang et al., 2004,

Sforza et al., 2012). This unicellular alga is recognized as an excellent source of eicosapentaenoic acid (EPA) which can be used for human consumption (Sukenik et al., 2009). *Nannochloropsis gaditana* is also considered as a precious pigments source in particular for carotenoids (Lubiàn et al., 2000, Forjan et al., 2007) and it is today used in aquaculture for the cultivation of fish (Cheng-Wu et al., 2001). In the last years there has also been an increased interest in the entire genus for biofuel production due to its particular high content in lipids which is for the majority composed of palmitic and palmitoleic acid (Gouveia et Oliveira, 2009, Sukenik et al., 2009). *Nannochloropsis*, as other algal species, has been shown to increase its lipid content in response to abiotic stresses such as nitrogen and/or phosphorous starvation in particular (Gouveia et Oliveira, 2009, Rodolfi et al., 2008). High light has been also suggested to increase the lipid accumulation (Damiani et al., 2010, Solovchenkov et al., 2010).

1.3. PHOTOSYNTHESIS: THE FUNDAMENTAL PROCESS FOR LIFE ON EARTH

Oxygenic photosynthesis is the process that allows plants, algae and some bacteria to convert solar light energy into chemical energy useful for the synthesis of ATP, reducing equivalents and organic compounds such as carbohydrates. This reaction is also responsible for the oxygen availability on Earth. This fundamental process can be divided in two parts: during the first one defined "light phase" light energy is used to oxidize water molecules and produce ATP and NADPH. These latter molecules are then used in the second "dark phase" for the carbon fixation whose products are then exploited for the synthesis of organic compounds and finally biomass (Takahaschi and Murata, 2008). The molecules responsible for the capture of light energy from the sun

are defined pigments and they are divided into two groups: chlorophylls and carotenoids.

1.3.1. Chlorophylls

Chlorophylls belongs to the class of metal porphyrins containing Mg^{2+} in the center of the tetrapyrrole ring. All kind of chlorophylls have a long lateral hydrophobic chain characterized by 20 carbon atoms which give a strong apolarity to the molecule (figure 4A).

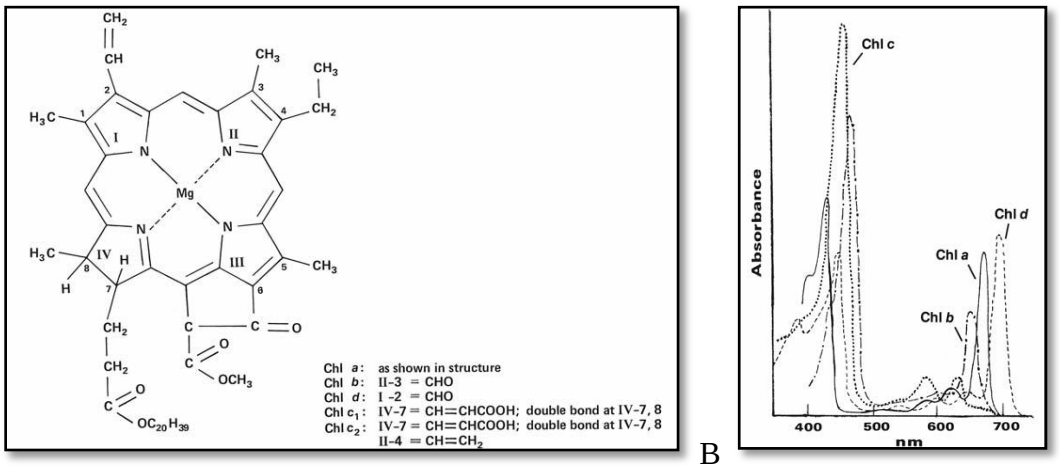

Figure 4. A) Chlorophylls structure. B) The absorption spectra of chlorophylls *a, b, c* and *d* (Lee et al., 2008).

In photosynthetic organisms chlorophyll (Chl) *a* is always present playing a central role in the harvesting and in photochemical transformation of the light energy. Other types of Chls (*b*, *c*, *d* and bacterio-chl) are also present, differently distributed in various organisms, with a major role in the light harvesting. All Chls efficiently absorb light expecially in the blue and red regions. Different Chls have a specific absorption range in the visible region of the light spectrum (figure 4B), due to the specific bounds in the tetrapyrrole ring. Plants and algae present some differences in the chlorophyll content. In fact higher plants (and green algae) possess only chl *a* and *b*, while in the very diverse group of algae are also present the other Chls following a species specific

distribution. Chlorophylls are often associated to proteins composing chl-protein complexes focused on the light capture (Nelson and Cox, 2000, Raven et al., 2002, Buchanan et al., 2003).

1.3.2. Carotenoids

Carotenoids (Car) (figure 5) are isoprenoid compounds with 40 carbon atoms which are normally associated to proteins. When carotenoids present at least one oxygen atom in the structure they are defined xanthophylls. Carotenoids absorb light in the range between 350 and 500 nm, where Chls are less efficient. Thus carotenoids increase plants and algae ability to absorb in the light spectrum, contributing to light harvesting. Carotenoids, however, also play a fundamental role in different photoprotective mechanisms from photoxidative damages quenching chl triplet states, scavenging reactive oxygen species and dissipating excess energy (Demmig-Adams et al., 1996, Niyogi et al., 1997, Taiz and Zeiger, 2006, Li et al., 2009). As in the case of chlorophylls, the distribution of different carotenoid molecules is diverse with species specific molecules identified in specific groups of organisms (Takaichi, 2011).

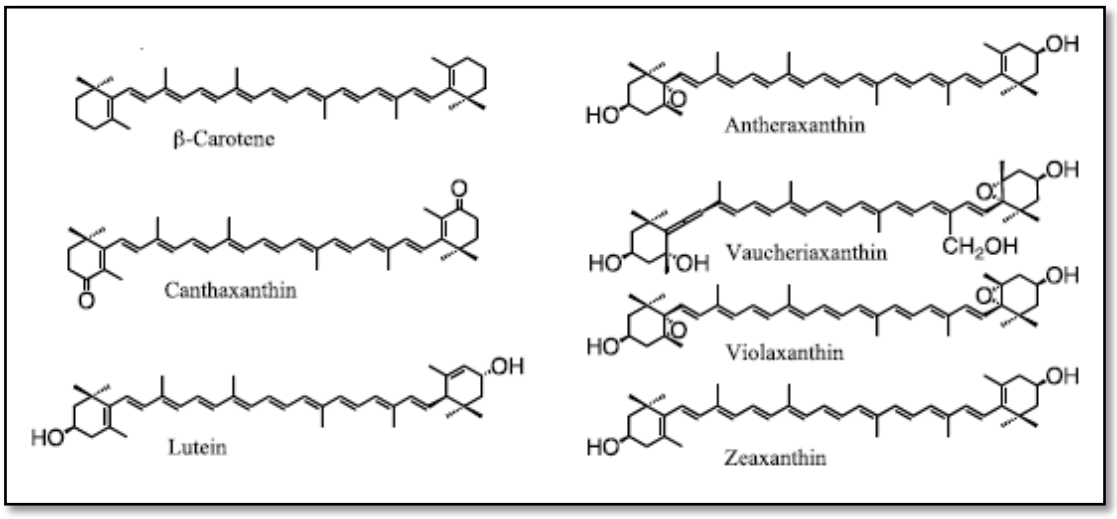

Figure 5. Structure of some carotenoids (Takaichi, 2011) often found in photosynthetic organisms.

1.4. THE BASE OF THE PHOTOSYNTHESIS: THE CHLOROPLAST

The mechanisms of capture, transfer and conversion of light energy into chemical energy require some particular structures that are localized in specific portions of the thylakoid membranes inside the chloroplast (figure 6). The chloroplast originated from a primary endosymbiontic event between an heterotrophic unicellular organism and a cyanobacterium (Parker et al., 2008) which determined the presence of two membrane in the chloroplast. However, other events of endosymbiosis allow the presence of two to four membranes that envelop the chloroplast (Yoon et al., 2002). The thylakoids inside the chloroplast are a complex system of membranes that could be individually organized delimiting the *stroma* or in stacked piles forming structures called *grana* (Taiz and Zeiger, 2006). Thylakoids present a peculiar composition due to the abundance of galactolipids as MGDG and DGDG but also lipid sulphur (SQDG) and phospholipid (PG) (Raven et al., 2002).

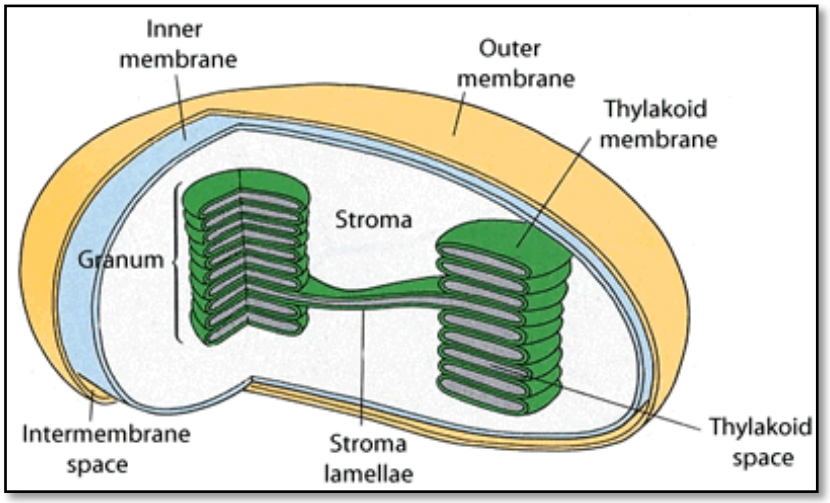

Figure 6. Chloroplast structure (http://liquidbio.pbworks.com).

1.5. THE ELECTRON TRANSPORT CHAIN: PHOTOCHEMISTRY REACTIONS

In the photochemistry reactions, following the linear mode (Linear Electron Flow, LEF), electrons are transferred from water to NADP via three major transmembrane complexes: Photosystem II (PSII), the cytochrome b_6f complex and photosystem I (PSI). NADPH so produced is used in the Calvin-Benson-Bassham cycle, where CO_2 is fixed to produce sugars (figure 7) (Joliot and Johnson, 2011). The two pigment-protein complexes called photosystems act in series and are able to catalyze the first step of the energy conversion, the light-induced charge separation. A photosystem is schematically composed of a light harvesting complex (LHC) and a reaction center (RC). Pigments in the LHC are responsible for the capture of photons, whose excitation energy is thus transferred to the base of the charge separation reaction, the RC. Two specific Chl *a* molecules in the RC are able to use excitation energy to transfer one electron to the following component of the photosynthesis chain.

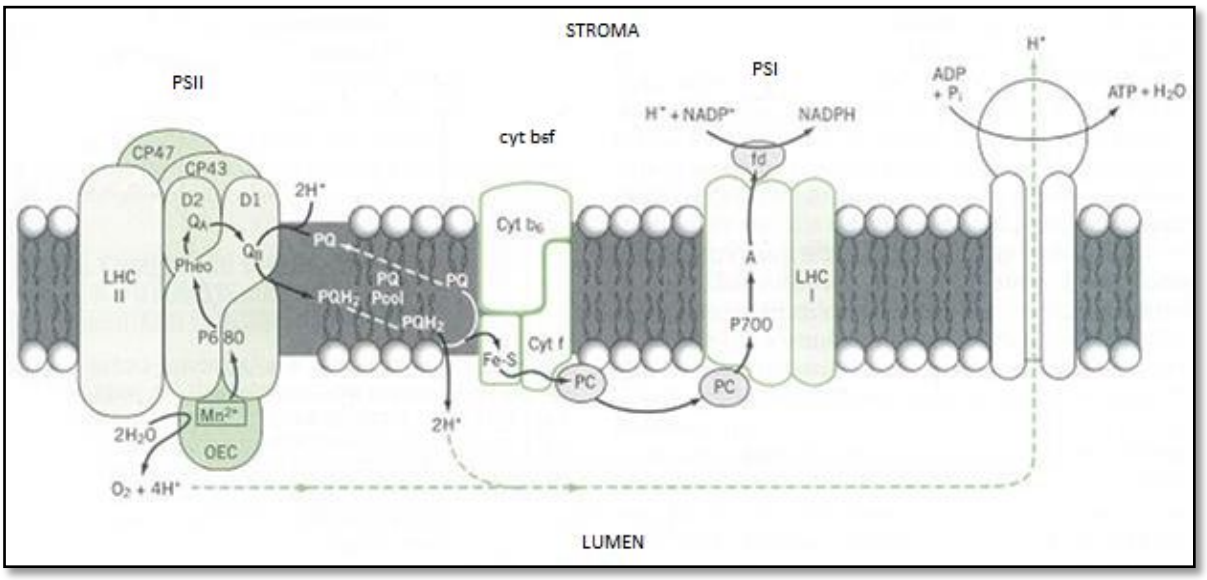

Figure 7. Electron transport chain in thylakoids (Hopkins and Huner, 2007). PSII and PSI photosystem II and I, cyt b_6f cytochrome b_6f complex, OEC oxygen evolving complex, PC plastocianin, FD ferredoxin, LHC light harvesting complex

In the case of PSII, the electron is transferred from the special Chl *a* dimer (P680) first to a pheophytin molecule. The electrons lost by P680 are replaced with others deriving from water thanks to the activity of the Oxygen Evolving Complex (OEC), which is responsible for the water photolysis. This process increases also the protons amount in the thylakoids lumen that could be used for the ATP synthesis by the activity of ATP synthase complex. Through the pheophytin and the plastoquinone pool (PQ), the electrons are transferred to the complex of cytochrome b_6f (Cyt b_6f) thanks to the reduction of PQ to PQH_2, which presenst less affinity for the PSII. From the Cyt b_6f complex electrons are then transferred to the Chl *a* dimer in the PSI, defined P700, through a soluble copper-protein, the plastocianin (Chylla and Whitmarsh, 1989, Hopkins and Huner, 2007, Joliot and Johnson, 2011). In some particular conditions such as copper deficiency, algae are able to substitute the PC with Cyt c_6, which has the same function (Inda et al., 1999, Crowley et al., 2002). On the stromal side, from the P700 the electrons are finally transferred trough an iron containing protein, the ferredoxin (FD), for the reduction of NADP in NADPH, afterward utilized in the Calvin cycle (Hopkins and Huner, 2007, Joliot and Johnson, 2011).

1.6. PHOTOSYNTHETIC APPARATUS COMPONENTS

1.6.1. Photosystem II (PSII)

The reaction center of PSII is composed of two proteins, D1 and D2, each containing five transmembrane α-helices, which bind the chlorophyll, pheophytin and plastoquinone co-factors involved in transmembrane light-induced charge separation. PSII has an inner light harvesting complex containing the proteins CP43 and CP47 with six transmembrane α-helices able to bind chlorophyll *a* and β-carotene (Hopkins and Huner, 2007). PSII is also prone to various types of light-induced irreversible damage,

which unless repaired would lead to an inhibition of PSII activity with a consequent reduction in oxygenic photosynthesis and growth (Nixon et al., 2010). In order to maintain efficient PSII complexes a repair cycle is required to replace damaged proteins, mainly the D1 (Kim et al., 1993) although upon illumination conditions other PSII subunits could be damaged such as D2 and CP43 (Nixon et al., 2005, Nixon et al., 2010). PSII is localized in the grana of thylakoids and it is strictly linked to the OEC in the luminal side of the membrane.

1.6.2. Photosystem I (PSI)

The reaction center of PSI is composed of many polypeptides. Among them, PsaA and PsaB bind the Chl *a* dimer P700, responsible for the charge separation reaction. The other polypeptides (PsaC, D and E) are involved in the link with the PC in the luminal side of the membrane or with FD in the stromal one. The inner light harvesting complex of PSI contains many Chl *a* molecules and β-carotene with the function of channel the energy to the RC (Fromme et al., 2001, Hopkins and Huner, 2007).

1.6.3. Light harvesting complex (LHC)

Photosystem II and I also include an external antenna system, composed by members of the multigenic family called LHC (Light Harvesting Complex). In contrast with the reaction center and the inner light harvesting system, the LHC complexes are not conserved during evolution and present a clear variability among different photosynthetic organisms (Koziol et al., 2007). In the model alga we chose for this work, *Nannochloropsis gaditana*, the major polypeptide of its antenna system was called violaxanthin–chl *a* binding protein (VCP). It binds only Chl *a* and violaxanthin (Sukenik et al. 1992, Sukenik et al. 2000), which acts as an antenna pigment for photosynthesis (Brown, 1987). This is different from plants and other algae antenna system, thus it only presents Chl *a*.

1.7. CYCLIC ELECTRON FLOW AROUND PSI: AN ALTERNATIVE TO THE LINEAR ELECTRON FLOW FROM PSII TO PSI

Plants and algae during the adaptation to unfavorable environments in terms of light and nutrient availability exploited the high flexibility of the photosynthetic machinery. Cyclic electron flow (CEF) around PSI, or cyclic photophosphorylation, is the photosynthetic process which recycles the reducing equivalents produced by PSI in the stroma towards the plastoquinone pool. Cytochrome b_6f complex is involved in CEF, also contributing to the transfer of protons across the membrane. CEF generates an electrochemical proton gradient across the thylakoid membrane without net production of reducing equivalents, thus promoting the synthesis of ATP but not NADPH (Alrich, 2010, Peltier et al., 2010, Joliot and Johnson, 2011). The linear electron transport from PSII to PSI alone was suggested to be insufficient for the generation of all the required ATP to balance the ATP:NADPH consumption rate in the Calvin cycle so one of the supposed function for the CEF is to support the extra ATP production (Peltier et al., 2010). Another major function for cyclic electron flow is presumed to be correlated with the activation of the Non Photochemical Quenching (NPQ) within the antenna, protecting PSII against excess light. In fact this mechanism of photoprotection requires a large proton gradient and the activation of cyclic electron transfer could provide an important contribution (Joliot and Johnson, 2011).

1.8. REFERENCE LIST

Alric, J. (2010). Cyclic electron flow around photosystem I in unicellular green algae. Photosynthesis Research.

Boussiba S., Vonshak A., Cohen Z., Avissar Y. and Richmond A. (1987). Lipid and Biomass Production by the halotolerant Microalga *Nannochloropsis salina.* Biomass.

Brown J. S. (1987). Functional Organization of Chlorophyll a and Carotenoids in the Alga, *Nannochloropsis salina*. Plant Physiology.

Buchanan B. B., Wilhelm J. and Russel L. (2003). *Biochimica* e biologia molecolare delle piante. Zanichelli Ed.

Chisti Y. (2008). Biodiesel from microalgae beats bioethanol. Trends in Biotechnology.

Chisti Y. (2010). Fuels from microalgae. Biofuels.

Chisti Y. and Yan J. Y. (2011). Energy from algae: current status and future trends. Algal biofuels – A status report. Applied Energy.

Cheng-Wu, Zmora O., Kopel R. and Richmond A. (2001). An industrial-size flat plate glass reactor for mass production of *Nannochloropsis sp.* (Eustigmatophyceae). Aquaculture.

Chylla R. A. and Whitmarsh J. (1989). Inactive Photosystem II complexes in leaves.

Crowley P. B., Diaz-Quintana A., Molina-Heredia F. P., Nieto P., Sutter M., Haehnel W., De la Rosa M. A. and Ubbink M. (2002). The Interactions of cyanobacterial Cytochrome *c*6 and Cytochrome *f*, characterized by NMR. The Journal of Biological Chemistry.

Damiani M. C., Popovich C. A., Constenla D. and Leonardi P. I. (2010) Lipid analysis in *Haematococcus pluvialis* to assess its potential use as a biodiesel feedstock. Bioresource Technology.

Del Campo J. A., García-González M. and Guerrero M. G. (2007). Outdoor cultivation of microalgae for carotenoid production: current state and perspectives. Applied Microbiology and Biotechnology.

Demmig-Adams B., Adams W.W., Barker D.H., Logan B.A., Bowling D.R. and Verhoeven A.S. (1996). Using chlorophyll fluorescence to assess the fraction of absorbed light allocated to thermal dissipation of excess excitation. Physiologia Plantarum.

Fang X., Wei C., Zhao-Ling C. and Fan O. (2004). Effects of organic carbon sources on cell growth and eicosapentaenoic acid content of *Nannochloropsis* sp. Journal of Applied Phycology.

Forjan I., Garbayo I., Casal C. and Vílchez C. (2007). Enhancement of carotenoid production in *Nannochloropsis* by phosphate and sulphur limitation. Communicating Current Research and Educational Topics and Trends in Applied Microbiology.

Fromme P., Jordan P. and Krauss M. (2001). Structure of photosystem I. Biochimica et Biophysica Acta.

Gouveia L. and Oliveira A. C. (2009). Microalgae as a raw material for biofuels production. Journal of Industrial Microbiology and Biotechnology.

Govoni C., Morosinotto T., Giuliano G. and Bassi R. (2008). Exploiting Photosynthesis for biofuel production. Biophotonics. Springel Ed. Verlag.

Green B. R. and Geider R. J. and Falkowski P. G. (1991). Effect of iron limitation on photosynthesis in a marine diatom. Limnology Oceanography.

Guedes A. C., Amaro H. M. and Malcata F. X. (2011). Microalgae as sources of carotenoids. Marine Drugs.

Hannon M., Gimpel J., Tran M., Rasala B. and Mayfield S. (2010). Biofuels from algae: challenges and potential. Biofuels.

Hibberd J. D. (1981). Notes on the taxonomy and nomenclature of the algal classes *Eustigmatophyceae* and *Tribophyceae* (synonym *Xanthophyceae*). Botanical Journal of the *Linnean Society*.

Hodgson P. A., Henderson R. J., Sargent J. R. and Leftley J. W. (1991). Patterns of variation in the lipid class and fatty-acid composition of *Nannochloropsis Oculata* (Eustigmatophyceae) during batch culture in the growth cycle. Journal of Applied Phycology.

Hopkins W. G. and Huner N. P. A., (2007). Fisiologia vegetale. Ed. McGraw-Hill.

Hu H. and Gao K. (2006). Response of growth and fatty acid compositions of Nannochloropsis sp. to environmental factors under elevated CO_2 concentration. Biotechnology Letters.

Inda L. A., Erdner D.L., Peleato M. L. and Anderson D. M. (1999). Cytochrome c_6 isolated from the marine diatom *Thalassiosira weissflogii*. Phytochemistry.

Jeong G. T., Park D. H., Kang C. H., Lee W. T., Sunwoo C. S., Yoon C. H., Choi B. C., Kim H. S., Kim S. W. and Lee U. T. (2004). Production of biodiesel fuel by transesterification of rapeseed oil. Applied Biochemistry and Biotechnology.

Jiang L., Luo S., Fan X., Yang Z. and Guo R. (2011). Biomass and lipid production of marine microalgae using municipal wastewater and high concentration of CO_2. Applied Energy.

Joliot P. and Johnson G. N. (2011). Regulation of cyclic and linear electron flow in higher plants. PNAS.

Kim J. H., Nemson J. A. and Melis A. (1993). Photosystem II reaction center damage and repair in *Dunaliella salina* (Green Alga). Analysis under physiological and irradiance stress conditions. Plant Physiology.

Koziol A.G., Borza T., Ishida K. I., Keeling P., Lee R. W. and Durnford D. G. (2007). Tracing the evolution of the light-harvesting antennae in chlorophyll a/b-containing organisms. Plant Physiology.

Kreimer G. (2009). The green algal eyespot apparatus: a primordial visual system and more? Current Genetics.

Lee R. E. (2008). Phycology Fourth edition. Cambridge University Press.

Li Z., Wakao S., Fischer B. B. and Niyogi K. K. (2009). Sensing and responding to excess light. Annual Review of Plant Biology.

Lubiàn L. M. (1982). *Nannochloropsis gaditana sp. nov*., una nueva eustigmatophyceae marina. Lazaroa.

Lubiàn L. M., Establier R. and Ruiz E. (1986). La luz y el nitrato en el crescimiento y contenido de pigmentos en cultivos de *Nannochloropsis gaditana Lubiàn*. Investigacion Pesquera.

Lubiàn L. M. (2000). *Nannochloropsis (Eustigmatophyceae)* as source of commercially valuable pigments. Journal of Applied Phycology.

Morosinotto T., Bonente G., Govoni C. and Bassi R. (2009) Biocombustibili da alghe unicellulari. Le piante industriali per la multifunzionalità e sostenibilità dell'agricoltura italiana nel terzo millennio. (Edizioni Ranalli).

Nelson D. L. and Cox M. M. (2000). Lehninger principles of biochemistry. Third edition. Worth publishers, Inc.

Nixon P. J., Barker M., Boehm M., de Vries R. and Komenda J. (2005). FtsH-mediated repair of the photosystem II complex in response to light stress. Journal of Experimental Botany.

Nixon P. J., Michoux F., Yu J., Boehm M. and Komenda J. (2010). Recent advances in understanding the assembly and repair of Photosystem II. Annals of Botany.

Niyogi K. K., Bjorkman O. and Grossman A. R. (1997). *Chlamydomonas* Xanthophyll Cycle mutants identified by video imaging of chlorophyll fluorescence quenching. The Plant Cell.

Parker M. S., Mock T. and Armbrust E. V. (2008). Genomic insights into marine microalgae. Annuals Review of Genetics.

Peltier G., Tolleter D., Billon E. and Cournac L. (2010). Auxiliary electron transport pathways in chloroplasts of microalgae. Photosynthesis Research.

Radakowitz R., Jinkerson R. E., Darzins A. and Posewitz M. C. (2010). Genetic engineering of algae for enhanced biofuel production. Eukariotic Cell.

Raven P. H., Evert R. F. and Eichorn S.E. (2002). Biologia delle piante. Sesta edizione. Ed. Zanichelli.

Riisberg I. (2009). Seven gene phylogeny of *Heterokonts*. Protist.

Rodolfi L., Chini Z.G., Bassi N., Padovani G., Biondi N., Bonini G. and Tredici M. R. (2008). Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnology and Bioengineering.

Santos L. M. A. and Leedale G.F. (1995). Some note on the ultrastructure of small azoosporic members of the algal class *Eustigmatophyceae*. Nova Hedwigia.

Sforza E., Bertucco A., Morosinotto T. and Giacometti G.M. (2010). Vegetal oil from microalgae: species selection and optimization. Chemical Engineering Transaction.

Sforza E., Cipriani R., Morosinotto T., Bertucco A. and Giacometti G. M. (2012). Excess CO_2 supply inhibits mixotrophic growth of *Chlorella protothecoides* and *Nannochloropsis salina*. Bioresource Technology.

Simionato D., Sforza E., Corteggiani Carpinelli E., Bertucco A., Giacometti G. M. and Morosinotto T. (2011). Acclimation of *Nannochloropsis gaditana* to different illumination regimes: effects on lipids accumulation. Bioresource Technology.

Sivakumar G., Xu J., Thompson R. W., Yang Y., Randol-Smith P. and Weathers P. J. (2012). Integrated green algal technology for bioremediation and biofuel. Bioresource Technology.

Solovchenkov A., Khozin-Goldberg I., Recht L. and Boussiba S. (2010). Stress-induced changes in optical properties, pigment and fatty acid content of *Nannochloropsis sp.:* implications for non-destructive assay of total fatty acids. Marine Biotechnology.

Sukenik A. and Livne A. (2000). Characterization of a gene encoding the light-harvesting violaxanthin chlorophil protein of *Nannochloropsis sp.* (Eustigmatophyceae). Journal of Phycology.

Sukenik A., Beardall J., Kromkamp J. C., Kopeck J., Masojídek J., Bergeijk S., Gabai S., Shaham E. and Yamshon A. (2009). Photosynthetic performance of outdoor *Nannochloropsis* mass cultures under a wide range of environmental conditions. Aquatic Microbial Ecology.

Sukenik A., Livne A., Neori A., Yacobi Y. Z. and Katcoff D. (1992). Purification and caracterization of a light-harvesting chlorophyll-protein complex from the marine Eustigmatophyte *Nannochloropsis sp.* Plant Cell Physiology.

Taiz L. and Zeiger E. (2006). Plant physiology. Fourth edition. Sinauer associated Inc. Publishers.

Takahaschi S. and Murata N. (2008). How do environmental stress accelerate photoinhibition?. Trends in Plant Science.

Takaichi S. (2011). Carotenoids in algae: distributions, biosyntheses and functions. Marine Drugs.

Takeshita T. (2011). Competitiveness, role, and impact of microalgal biodiesel in the global energy future. Applied Energy.

Yoon H.S., Hackett J.D., Pinto G. and Bhattacharya D. (2002). The single, ancient origin of chromist plastids. PNAS.

CHAPTER 2

ACCLIMATION OF *NANNOCHLOROPSIS GADITANA* TO DIFFERENT ILLUMINATION REGIMES: EFFECTS ON LIPIDS ACCUMULATION

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2.1. ABSTRACT

Algae are interesting potential sources of biodiesel, although research is still needed to develop efficient large scale productions. One major factor affecting productivity is light use efficiency. The effect of different light regimes on the seawater alga *Nannochloropsis gaditana* was accessed monitoring growth rate and photosynthetic performances. *N.gaditana* showed the capacity of acclimating to different light intensities, optimizing its photosynthetic apparatus to illumination. Thanks to this response, *N.gaditana* maintained similar growth rates under a wide range of irradiances, suggesting that this organism is a valuable candidate for outdoor productions in variable conditions. In the conditions tested here, without external CO_2 supply, light intensity alone was not found to be a major signal affecting lipids accumulation showing the absence of a direct regulatory link between the light stress and lipids accumulation. Strong illumination can nevertheless indirectly influences lipid accumulation if combined with other stresses or in the presence of excess CO_2.

2.2. INTRODUCTION

Microalgae are receiving a growing attention for their possible exploitation for biofuels production. In fact, several species of this group are able to accumulate large amounts of lipids and can be a suitable feedstock for biodiesel production. Currently biodiesel is obtained from oil rich seeds, but this process, due to the feedstock limitation, has little chance to be able to replace a significant fraction of fossil fuels (Singh et al., 2011). In the case of algae, productivities are estimated to be around ten times the one of crops, making these organisms a more promising source for biomass production on a long term perspective (Chisti, 2007; Chisti, 2008). Algae are a group of organisms with a very

large biological variability and thus with very different properties. Among them, the ones belonging to the genus *Nannochloropsis* are particularly interesting because of their ability to accumulate large amounts of lipids, which can reach concentrations up to 65-70% of total dry weight (Boussiba et al., 1987; Hodgson et al., 1991; Rodolfi et al., 2009). Such a massive accumulation of lipids was shown to be activated during the stationary phase of growth in response to stresses such as nitrogen and/or phosphorous starvation (Gouveia and Oliveira, 2009; Rodolfi et al., 2009).

In order to exploit these organisms for large scale biofuel production, it is fundamental to optimize productivity by obtaining a better understanding of the parameters influencing growth, biomass and lipids accumulation. As for all photosynthetic organisms, light is a major factor to be considered as it provides all the energy supporting metabolism. Radiation, however, can also be dangerous because, if in excess, it may drive the formation of reactive oxygen species[1] (ROS) and oxidative stress (Li et al., 2009). When cells are exposed to illumination, one component of the photosynthetic apparatus, photosystem II (PSII), is continuously damaged and inactivated in a well known process called photoinhibition (Murata et al., 2007). In order to maintain photosynthetic efficiency this complex must be continuously repaired by re-synthesis of damaged components (Nixon et al., 2010). Besides repairing the photoinhibitory damage, photosynthetic organisms have also evolved mechanisms to optimize light harvesting efficiency to physiological needs so as to prevent ROS formation and photoinhibition. Among these is light acclimation, where the composition of the photosynthetic apparatus is optimized according to irradiance intensity (Falkowski and LaRoche, 1991; Falkowski and Owens, 1980; Walters, 2005; Zou and

Richmond, 2000). Photoinhibition and its repairing are energetically very demanding and may strongly influence the biomass productivity. The capacity for an effective and prompt acclimation is therefore an important parameter to be considered in the characterization of an algal species for biomass production. In addition to its metabolic role, light is also known to play a large effect in signaling and its intensity affects many cellular processes (Brautigam et al., 2009; Eberhard et al., 2008; Kim et al., 2008).

The light intensity has also been suggested to influence algae capacity for lipids accumulation, which would be enhanced under strong illumination (Damiani et al., 2010). Such a phenomenon has been observed also for *Nannochloropsis* species, where a transition from control to high light conditions was found to induce a higher level of lipids accumulation (Fisher et al., 1998; Solovchenko et al., 2010).

In this work, the acclimation capacity of *Nannochloropsis gaditana* to different light regimes was analyzed, showing that during the exponential growth phase this organism is able to adapt to a wide range of light intensities by activating an acclimative response, as evidenced by the functional characterization of its photosynthetic apparatus. The light intensity *per se* does not appear to directly induce lipids accumulation, but excess illumination may enhance the effect of other stresses.

2.3. MATERIAL AND METHODS

2.3.1. Culture conditions

Nannochloropsis gaditana from CCAP, strain 849/5, was always grown in sterile filtered F/2 medium (Guillard and Ryther, 1962), using sea salts 32 g/l from SIGMA, 40 mM TRIS HCl pH 8, SIGMA Guillard's (F/2) marine water enrichment solution 1x.

Maintenance and propagation of cultures were performed using the same medium added with 10 g/l of Plant Agar (Duchefa Biochemie). Growth experiments were performed in Erlenmeyer flasks with magnetic agitation, starting from a pre-culture grown at 100 μE $m^{-2}s^{-1}$ at exponential phase which was diluted to an Optical Density (OD) value at 750 nm equal to 0.2, final volume 250 ml. Illumination was constant and intensities ranged from 5 to 2100 μE $m^{-2}s^{-1}$, using daylight fluorescent lamps (5-200 μE $m^{-2}s^{-1}$) or a LED Light Source SL 3500 (Photon Systems Instruments, 450-2100 μE m^{-2} s^{-1}). Temperature was kept at 23 ± 1 °C in a growth chamber. No external CO_2 supply was provided and thus carbon dioxide to support growth derived from the atmosphere. Algal growth was measured by daily changes in optical density OD_{750} determined spectrophotometrically with a Lambda Bio 40 UV/VIS Spectrometer (Perkin Elmer) and cells number monitored with a Bürker Counting Chamber (HBG, Germany) under light microscope. In the logarithmic growth phase cells number was related to optical density. The specific growth rate was calculated by the slope of logarithmic phase for number of cells. All curves were repeated at least three times and in all repetitions one culture at 100 μE $m^{-2}s^{-1}$ was always present as control.

2.3.2. Pigments extraction and analysis

Chlorophyll a and total Car were extracted from *Nannochloropsis* centrifuged cells at 4°C with 100 % N,N'-dimethylformamide for at least 48 hours in dark conditions as in (Moran and Porath, 1980). The pigments concentrations were determined spectrophotometrically using specific extinction coefficients (Porra et al., 1989; Wellburn, 1994).

2.3.3. In vivo monitoring of photosynthetic parameters

Chlorophyll fluorescence was determined in vivo using Dual PAM 100 from WALZ. The parameters Fv/Fm, NPQ and qP were calculated respectively as (Fm-Fo)/Fm, (Fm-Fm')/Fm' and (Fm'-F)/(Fm'-Fo) (Demmig-Adams et al., 1996) after 20 minutes of dark adaptation.

2.3.4. RuBIsCO content analysis

SDS-PAGE analysis was performed with a Tris–Glycine buffer system as in (Laemmli, 1970) with 12 % acrylamide.

2.3.5. Lipids analysis

The lipid content was determined by staining 2 millions of algal cells centrifuged and resuspended in 1.9 ml of de-ionized sterile water with Nile Red (NR) dye at final concentration of 2.5 µg/mL, for 10 minutes at 37°C (Chen et al., 2009). The fluorescence was measured using a spectrofluorometer (OLIS DM45), with excitation wavelength at 488 nm and emission wavelength in the range of 500 and 700 nm (Greenspan et al., 1985). The relative fluorescence of Nile Red for the lipids was obtained after subtraction of the autofluorescence of algal cells and Nile Red alone. Total lipids were extracted from dried cells using ethanol-hexane (2.5:1 vol/vol) as solvent in a Soxhlet apparatus for 10 h. The lipid mass was measured gravimetrically after solvent removal by a rotary evaporator. The fluorescence intensity of cells stained by NR is linearly correlated to the gravimetric ratio of cellular lipid.

2.4. RESULTS AND DISCUSSION

2.4.1. Effect of different irradiances on N. gaditana growth

Light has multiple effects on photosynthetic organisms, it provides the energy to support metabolism but it is also a fundamental signal influencing many cellular processes. In order to investigate light influence on *Nannochloropsis* growth and lipids accumulation one preliminary choice to be made was if providing externally carbon dioxide in excess or rely on the smaller amount present in the atmosphere. In the former case photosynthesis rate is increased while the latter is closer to environmental conditions. The aim of the work was not to achieve the maximal productivity but to investigate the regulatory connection between light intensity and the activation of lipid biosynthesis. In order to analyze regulation effects the conditions closer to a natural environment were chosen as more significant and thus experiments were performed with atmospheric CO_2 concentration.

Nannochloropsis gaditana was grown in batch cultures exposed to a wide interval of light conditions, ranging from very low to very high, respectively 5, 15, 50, 100, 200, 450, 1200, 2100 $\mu E\ m^{-2}s^{-1}$. In all cultures growth kinetics were monitored following the increase in cells number, as shown in figure 1. Data presented show that growth rates are remarkably similar for most light intensities tested and significant differences are observed only for the extreme conditions (5, 1200 and 2100 $\mu E\ m^{-2}s^{-1}$). In particular, cultures exposed to very low light showed slower growth, clearly imputable to a limitation in the energy available to support metabolism. On the other extreme, cultures exposed to very strong light reached the stationary phase earlier and final cells number was lower. The specific growth rate (μ) has very similar values for all curves but in the

case of that at 5 μE $m^{-2}s^{-1}$ (Figure 1D). Growth rates are similar to control also in the case of cultures exposed to the highest illumination (1200 and 2100 μE m^{-2} s^{-1}), although in this case experimental deviation is higher because values are calculated with a lower number of data points because of a shorter exponential phase.

Such a small effect of illumination on growth kinetics suggests that in these conditions, the duplication rate of *N. gaditana* is not light limited but depends on other factors like CO_2 and nutrient availability. It should be reminded, in fact, that carbon dioxide to support the growth was coming from the atmosphere and thus it is limiting at least in some illumination conditions. Nevertheless, these results suggest that the microalga *N. gaditana* has a remarkable capacity of acclimating to different photon fluxes by adapting its photosynthetic apparatus.

It is worth underlining that the observed capacity of maintaining the same growth rate in a wide range of light conditions is a valuable property in the perspective of using this species for biofuels production. In fact, in an outdoor environment light intensity is highly variable and thus any large scale application requires the chosen organism to be able to grow efficiently in a wide range of conditions.

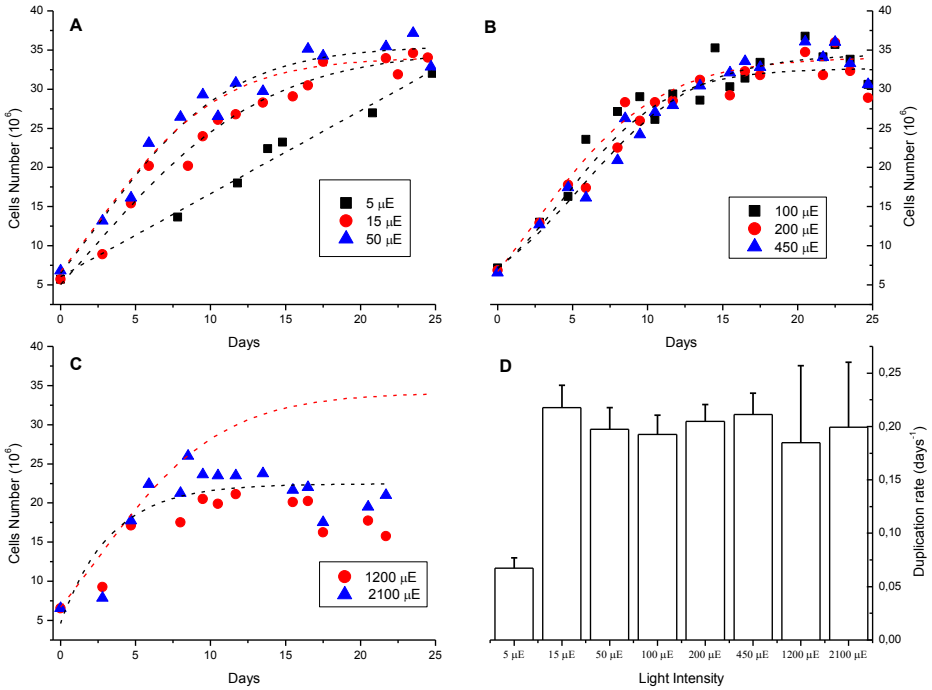


Figure 1. ***Nannochloropsis gaditana*** **growth curve under different light regimes.** A) Increase in cells number for illumination of 5, 15 and 50 µE (black squares, red circles and blue triangles respectively). Fitting the experimental points are also shown as dashed lines. Fitting of the growth curve at 100 µE is reported in red in all panes as reference. B) Growth kinetics with illumination at 100, 200 and 450 µE (black squares, red circles and blue triangles respectively). C) Curves with illumination at 1200 and 2100 µE (red circles and blue triangles respectively). D) Specific growth rate during exponential phase (µ) calculated for all growth curves. In this figure three weeks of cultivation are shown; while in many conditions stationary phase was prolonged, cultures at 2100 µE after three weeks bleached. All data are the result of merging at least three independent experiments.

2.4.2. Photosynthetic parameters under different photon fluxes

In order to access how photosynthetic apparatus responded to different light regimes, the fluorescence parameter Fv/Fm, a classical method to evaluate photosynthetic activity, was monitored (Maxwell and Johnson, 2000). Its value, in fact, represents a measure of PSII quantum yield and allows evaluating the presence of photo-induced damage to this protein complex, known to be the major target of photoinhibition

(Murata et al., 2007). This parameter showed a conserved behavior during the growth curve at all light regimes. During the exponential phase of the growth curve, Fv/Fm was stable (see Figure 2A) and average values were very similar for all light intensities, with the only exception of the highest one (2100 μE $m^{-2}s^{-1}$, Figure 2B), where a significant reduction was a clear indication of partial PSII photoinhibition. These results suggest that during the exponential phase this alga is able to deal with the light intensity and only the strongest illumination, in the order of the maximal intensity possibly found in natural conditions, induced measurable photoinhibition (figure 2).

Once the growth curve reached stationary phase cells are still photosynthetically active for several days although light energy is not exploited anymore for increasing the number of cells. During the stationary phase, however, photochemical activity decreased steadily in all cultures. This decrease was slow in the case of low light intensities where algae maintained a substantial photochemical efficiency for several weeks. With stronger illuminations the decline in photochemical efficiency was instead faster and with the strongest light intensity (2100 μE m^{-2} s^{-1}) after three weeks PSII activity was not anymore detectable (figure 2A). In following days these cells were rapidly bleached. These results suggest that at this stage cells are more sensitive to photoinhibition. This phenomenon can be attributed to a limitation in PSII repair which requires continuous protein turnover (Nixon et al., 2010) and thus is expected to be strongly affected by nutrients limitation. Thus, in the perspective of exploiting this alga for a large scale cultivation, in order to avoid the limitation in productivity imposed by photoinhibition, cells should be maintained in a state of active duplication.

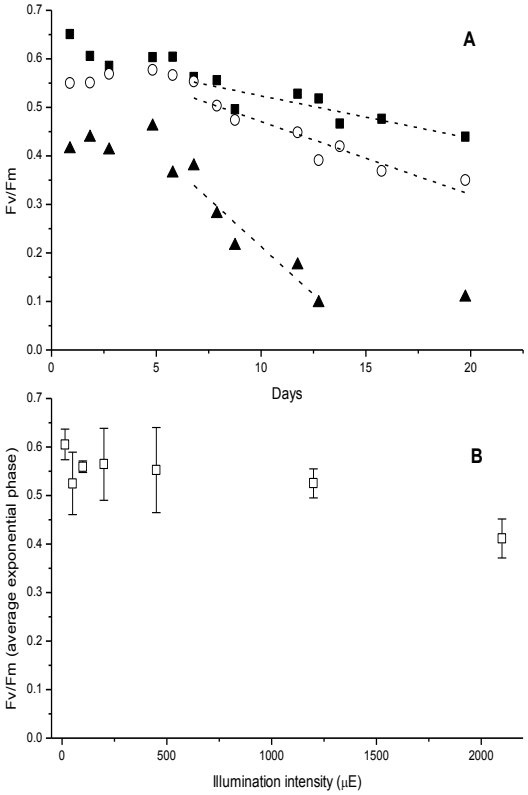


Figure 2. Photosynthetic parameters in *Nannochloropsis* cells acclimated to different light conditions. A) During growth curves reported in figure 1 Photosystem II efficiency was also monitored by determining the fluorescence parameter Fv/Fm. Here data for two extreme (15, 2100 μE, black squares and triangles respectively) and one intermediate illumination intensities (100 μE, open circles) are reported. The tendency during stationary phase is underlined by a dashed line. B) Average and Standard Deviation of Photosystem II efficiency (Fv/Fm) measured in different cultures during exponential phase (0-7 days; 0-5 in the case of 1200/2100 μE cultures) in dependence from the growing light intensity.

2.4.3. Photosynthetic acclimation: modulation of photochemical capacity and pigments content

Normally, photosynthetic organisms exposed to variable light conditions optimize their photochemical activity to the average illumination they are exposed to (Walters, 2005). qP (Photochemical Quenching) is a fluorescence parameter indicating the quantum yield

for photochemistry (Baker, 2008; Maxwell and Johnson, 2000) which allows evaluating if *N. gaditana* cultures modulated their photochemical efficiency in different growing conditions. When all absorbed energy is exploited for photochemistry qP is one, while when photosynthesis is saturated qP goes to zero. In order to verify if the quantum yield for photochemistry was varying in cultures acclimated to different illuminations, qP dependence from actinic light intensity in cultures acclimated to different light regimes was analyzed. Data in figure 3A clearly suggest that cultures grown with strong light (1200, 2100 μE $m^{-2}s^{-1}$) have higher qP with respect to the those grown with lower intensities (grown at 15 or 100 μE $m^{-2}s^{-1}$). In the latter, photosynthesis is saturated by lower photon fluxes. This result clearly suggests that cells adapted to high light can exploit a higher fraction of incident light for photochemistry with respect to cells acclimated to low light.

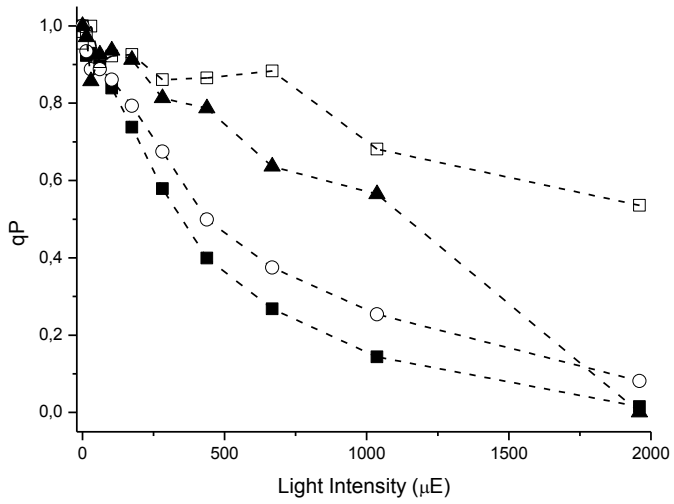


Figure 3A. Photochemical efficiency in *Nannochloropsis* acclimated cells. qP was measured in cells from the late exponential phase first dark adapted (20 min) and then treated with increasing actinic light (30 seconds for each point). After light treatment qP was determined by a saturating Flash. Here data for three extremes (15, 1200, 2100 μE) and one intermediate light intensities (100 μE) are reported. Data for cells grown at 15, 100, 1200, 2100 μE are shown as black squares, open circles, black triangles and open squares, respectively.

Such a modulation of the photochemical efficiency is often achieved by regulating the expression of the enzymes of the Calvin-Benson cycle, where CO_2 fixation occurs and most of the ATP and NADPH is consumed. In particular, the enzyme RuBisCO catalyzes the rate limiting step for carbon fixation and its cellular content determines the efficiency of the whole cycle. RuBisCO content was observed to be regulated by illumination conditions in several photosynthetic organisms (Fisher et al., 1989). RuBisCO content in *N. gaditana* cultures adapted to different light intensities was analyzed by using a specific antiserum recognizing its major subunit. As shown in the Western blot reported in figure 3B, the signal increases with the light intensity (with the exception of the case of 2100 μE $m^{-2}s^{-1}$), supporting the idea that this algal species is indeed capable of acclimating to different light conditions by modulating their photochemical capacity according to light conditions.

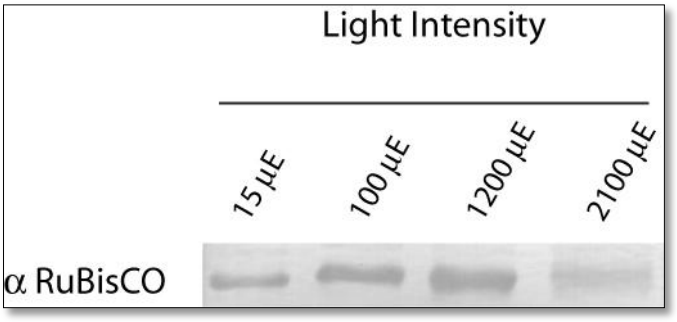


Figure 3B. Photochemical efficiency in *Nannochloropsis* acclimated cells. Evaluation of RuBisCO content in the same cells, determined using a specific antibody against the protein large subunit. Cellular extracts were loaded containing 1 Chl μg.

During acclimation, photosynthetic organisms often regulate their pigments content. Cellular chlorophyll (Chl) content in *N. gaditana* was strongly influenced by the illumination regime. Low light grown cells accumulated more chlorophylls than cells

adapted to high light and at the end of the exponential growth phase (data reported in table 1) Chl content was four times higher in low light (15 μE $m^{-2}s^{-1}$) than in high light cells (2100 μE $m^{-2}s^{-1}$). While Chls are essential for light harvesting, carotenoids are fundamental for protection from oxidative stress (Li et al., 2009). Consistently with their role in protection against ROS, carotenoids content was found to increase (relatively to Chl) under strong light (table 1). Taken together, these data clearly indicate that *N. gaditana* cells acclimate to different light regimes by accumulating different amount of pigments, Chls when light harvesting is limiting and carotenoids when absorbed energy is in excess and they need protection from photo-oxidation.

Table 1. Pigments content in *Nannochloropsis* acclimated cells. Chl content per cell and Car / Chl (μg/μg) ratio in cultures grown in different illumination regimes at the end of exponential phase (day 5) are reported.

Illumination Intensity (μE m^{-2} s^{-1})	**μg Chl / 10^6 cell**	**Car /Chl**
15	0.178 ± 0.025	0.33 ± 0.01
100	0.119 ± 0.007	0.40 ± 0.02
1200	0.067 ± 0.022	0.50 ± 0.02
2100	0.038 ± 0.002	0.55 ± 0.04

During acclimation, photosynthetic organisms adopt two strategies for modulate light harvesting efficiency and their pigment content: the first one is the increase of the antenna size, i.e. the number of chlorophyll/proteins of the light harvesting system associated to each reaction center; the second is increasing the number of reaction centers per cell (Falkowski and Owens, 1980; Walters, 2005). The functional PSII antenna size in *N. gaditana* was estimated by measuring fluorescence kinetics in cells treated with the inhibitor DCMU, which directly depends on the number of chlorophylls associated to each photosystem. As shown in Supplementary Figure S1 (see par. 2.6.)

very similar fluorescence time course was observed in cells grown with different illumination suggesting that the number of pigments transferring energy to each photosystem was stable. This is different from what is generally observed in both plants and algae where antenna size is modulated according to growth illumination but it is consistent with some previous reports on *Nannochloropsis* (Fisher et al., 1996; Fisher et al., 1998) and suggests that the increase in chlorophyll content upon acclimation to low light intensity is due to differential accumulation of photosystems rather than to an increased antenna size.

2.4.4. Activation of Non Photochemical Quenching

An important protection mechanism for photosynthetic organisms is the Non Photochemical Quenching (NPQ). This is a short-time response to excess energy which triggers thermal dissipation of excitation energy absorbed by light harvesting system before this hits the reaction center. This avoids ROS (mainly $^1O_2$) formation at the reaction center which would be produced by charge recombination in condition of over-reduction (Li et al., 2009). NPQ extent can be easily measured *in vivo* by fluorescence (Maxwell and Johnson, 2000) and it is modulated according to light acclimation in several organisms, both plants and algae (Ballottari et al., 2007; Peers et al., 2009). To access if NPQ response was modified in different cultures, NPQ was measured for each culture as a function of the actinic light intensity (figure 4). Cells grown at 15-100 μE $m^{-2}s^{-1}$ show a similar behavior, with NPQ activated at very low illumination, and with a tendency to saturate at higher intensities. This suggests that *N. gaditana,* at difference with other algae such as *Chlamydomonas reinhardtii* (Peers et al., 2009), is constitutively able to induce NPQ, even if acclimated to very low light. In cultures

acclimated to stronger light regimes (1200 and 2100 μE $m^{-2}s^{-1}$), dim actinic lights induce relatively lower NPQ levels. In fact, as previously observed, these cells are able to use light for photochemistry with higher efficiency, and this reduces the need for energy dissipation. When actinic light increases and saturates the photochemical capacity of the cell, NPQ is activated and reaches quite high values. In the case of cells acclimated at 2100 μE $m^{-2}s^{-1}$, NPQ saturation was not attained even at the maximum of the actinic light intensity employed. Taken together, these results show that, in *N. gaditana,* acclimation can actually modulate also NPQ, adjusting its level according to its different needs to dissipate the energy absorbed by the light harvesting system.

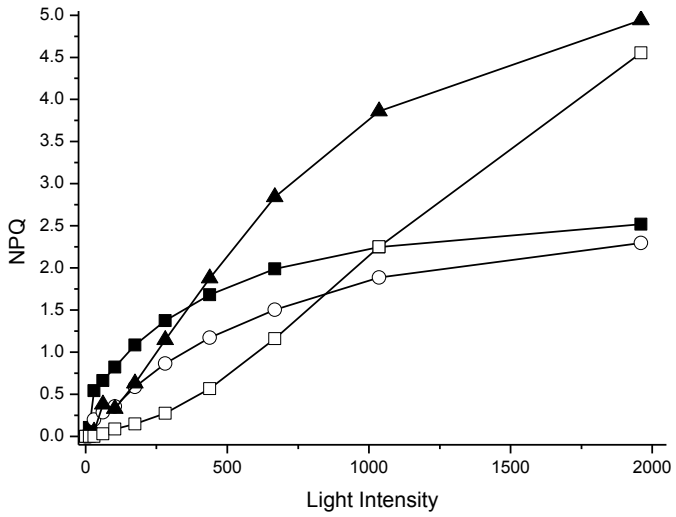


Figure 4. Non Photochemical Quenching in *Nannochloropsis* acclimated cells. NPQ dependence on actinic light intensity was measured in cells acclimated to different light conditions for 5 days. Data at 15, 100, 1200, 2100 μE m^{-2} s^{-1} are shown as black squares, open circles, black triangles and open squares, respectively. 30 seconds of actinic light were used to induce NPQ.

2.4.5. Nannochloropsis lipids accumulation on different light conditions

As mentioned, *Nannochloropsis* has a remarkable ability of accumulating lipids in certain conditions. When grown in a relatively poor substrate as F/2, cells experience

nutrients limitation and in the stationary phase they accumulate large amounts of lipids (Hodgson et al., 1991; Rodolfi et al., 2009; Sforza et al., 2010). An important question for the characterization of an algal species for biofuels production is whether cellular lipids accumulation is affected by exposure to different light regimes. To address this question the non polar lipids content was evaluated in cultures grown at different light intensity by Nile Red staining (Chen et al., 2009; Sforza et al., 2010). During the exponential growth phase at all light regimes only very small signals, corresponding to the constitutive cellular lipids, were detectable. During the stationary phase, lipids started accumulating as evidenced by the strong increase of the fluorescence signal (Figure 5). The extent of lipids accumulation is very similar for all irradiances but for 1200 μE m^{-2} s^{-1} cultures where their synthesis started earlier. In a later phase of the curve other differences emerged. Cells with the lower illumination continued accumulating lipids, reaching after 4 weeks, a fluorescence value per 10^6 cells corresponding to around 50% of total dry-weight, as determined by gravimetric standardization (Sforza et al., 2010). On the contrary, in cells exposed to 1200 μE m^{-2} s^{-1} lipids accumulation did not increase further reaching saturation. Finally, in cultures with the strongest illumination (2100 μE m^{-2} s^{-1}) lipids synthesis was initially induced but afterwards cells bleached out and no lipids were detectable after three weeks. These results suggest that the major signal inducing lipids accumulation was nutrients starvation during the stationary phase and that light intensity *per se* did not have an influence on lipids accumulation.

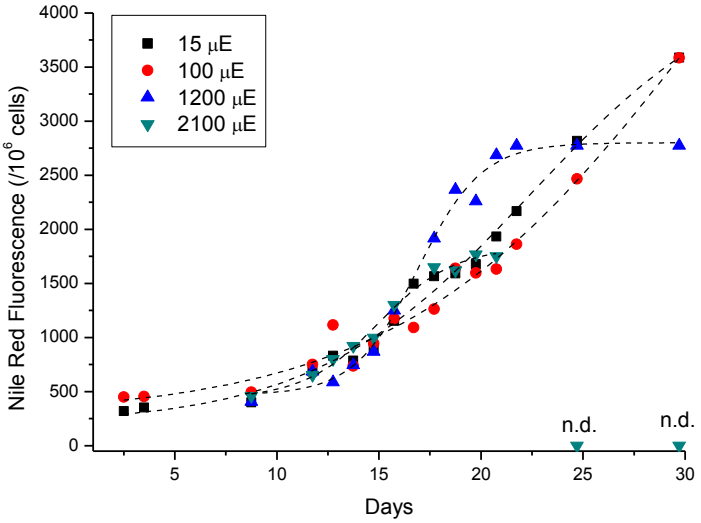


Figure 5. Lipids accumulation under different illumination. Lipids were quantified from the fluorescence at 585 nm of Nile Red stained cells. For culture at 2100 µE m^{-2} s^{-1} after three weeks, fluorescence signal was not detectable anymore.

It is worth reminding that in the cultures analyzed here carbon dioxide is likely limiting for growth at least in some of the conditions tested. By providing excess CO_2 light effect might be different and future experiments will clarify this point. Nevertheless, the present study clearly shows that light intensity alone does not regulate lipids accumulation. Thus, molecular regulatory networks responding to light signals do not influence directly the regulation of lipid biosynthesis as the nitrogen availability does. Light is in fact known to activate many molecular signaling networks which respond for instance to electron transporters redox state or the accumulation of different ROS species (Brautigam et al., 2009; Eberhard et al., 2008; Kim et al., 2008). This work clearly suggest that these signaling networks are not involved in regulation of lipids biosynthesis in *Nannochloropsis*. Illumination conditions might play an indirect but still important role if combined with other factors influencing photosynthesis and growth like CO_2 supply.

Presented data also show that illumination also indirectly influences nutrients availability since strong light might require intense repair of photosynthetic apparatus and thus higher nutrients consumption. This is likely what happened with 1200 μE m^{-2} s^{-1} cultures for which lipids biosynthesis started earlier, probably because of a precocious transition to the stationary phase.

This relatively low effect of illumination on lipids accumulation is in apparent contrast with other results (Packer et al., 2011; Solovchenko et al., 2010) showing that a light stress enhanced lipids biosynthesis. When comparing results it should be considered than in these previous cases CO_2 was supplied in excess which, as discussed above, might affect the results. Another factor to be considered is that, experimental design was also different : while in these previous works cells were suddenly exposed to a stronger illumination, here cultures were grown at different illumination from the beginning. As showed above, in our case the constant exposition to different irradiances allowed the activation of an acclimative response and optimization of photosynthetic apparatus to illumination. Thus, considering the influence of light on the lipids productivity of *N. gaditana,* three different cases can be distinguished (figure 6): In the first scenario, cells are exposed to a constant low or moderate light and in this case lipids accumulation is activated by nutrients limitation during the stationary phase. In a second case, cells grow at moderate intensity but are subjected to a light stress during the stationary phase. In this case, the irradiance changes in a phase where cells are prone to photoinhibition, fortifies nutrients deprivation stress and enhances lipids accumulation. The third case is the one of a constant exposition to a strong irradiance. In this case, cells are capable of acclimating to the high light by enhancing protective mechanisms and optimizing their photosynthetic apparatus. Thanks to this response, in the stationary phase the light stress

perceived is lower with small effect on lipids production which is again induced by nutrients deprivation.

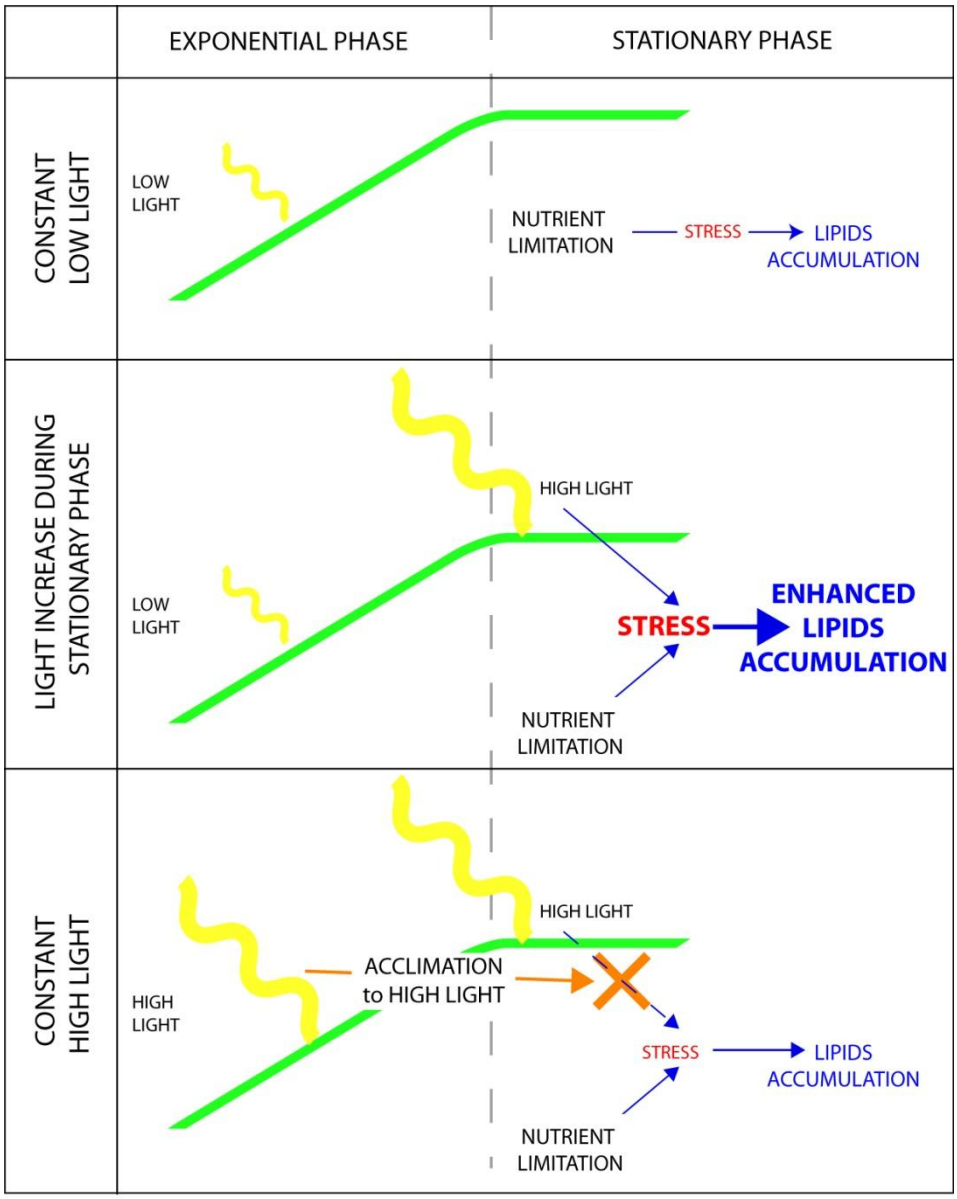


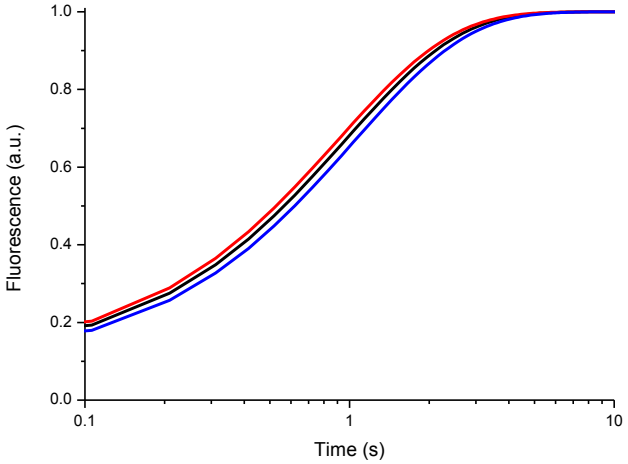
Figure 6. Scheme of light effect on lipids accumulation in *Nannochloropsis*. N. *gaditana* grown in batch under low light shows a reproducible behavior. During the stationary phase, the stress due to nutrients deprivation induces lipids biosynthesis. A sudden increase of the illumination intensity during the stationary phase enhances nutrients deprivation and lipids accumulation. If cells are grown in strong light, activation of the acclimation response decreases the light induced stress on the cells, with no enhancement of lipids accumulation.

2.5. CONCLUSIONS

This work showed that *N. gaditana* is capable of optimizing its photosynthetic apparatus to variable illumination conditions, suggesting this species is a valuable candidate for biomass production in an outdoor environment.

N. gaditana cells accumulate lipids in response to nutrients limitation: during this growth phase cells are photosynthetically active but use light energy to synthesize lipids instead of sustaining cell duplication. At this stage, however, cells are more prone to radiation damages if exposed to intense light. Exposure to strong light alone did not induce lipids accumulation in this species but illumination may fortify signals from nutrients deprivation.

2.6. SUPPLEMENTARY FIGURES



Supplementary Figure S1. Antenna size measured on *Nannochloropsis* cells acclimated to different light conditions. Antenna size was measured in *Nannochloropsis* cells grown in different light conditions by measuring fluorescence rise in cells treated one hour with DCMU (final concentration 90 µM). Curves measured for cells grown at 15, 100 and 1200 µE are shown in blue, red and black respectively. Antenna size can be estimated by the rising time $t_{2/3}$. Fluorescence is normalized to maximum value.

2.7. REFERENCE LIST

Baker N.R. 2008 Chlorophyll fluorescence: a probe of photosynthesis in vivo. Annu. Rev. Plant Biol. 59, 89-113.

Ballottari M., Dall'Osto L., Morosinotto T. and Bassi R. 2007 Contrasting behavior of higher plant photosystem I and II antenna systems during acclimation. J. Biol. Chem. 282, 8947-8958.

Boussiba S., Vonshak A., Cohen Z., Avissar Y. and Richmond A. 1987 Lipid and Biomass Production by the Halotolerant Microalga Nannochloropsis-Salina. Biomass 12, 37-47.

Brautigam K., Dietzel L., Kleine T., Stroher E., Wormuth D., Dietz K.J., Radke D., Wirtz M., Hell R., Dormann P., Nunes-Nesi A., Schauer N., Fernie A.R., Oliver S.N., Geigenberger P., Leister D. and Pfannschmidt T. 2009 Dynamic plastid redox signals integrate gene expression and metabolism to induce distinct metabolic states in photosynthetic acclimation in Arabidopsis. Plant Cell 21, 2715-2732.

Chen W., Zhang C., Song L., Sommerfeld M. and Hu Q. 2009 A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. J. Microbiol. Methods 77, 41-47.

Chisti Y. 2007 Biodiesel from microalgae. Biotechnol. Adv. 25, 294-306.

Chisti Y. 2008 Biodiesel from microalgae beats bioethanol. Trends Biotechnol. 26, 126-131.

Damiani M.C., Popovich C.A., Constenla D. and Leonardi P.I. 2010 Lipid analysis in Haematococcus pluvialis to assess its potential use as a biodiesel feedstock. Bioresour. Technol. 101, 3801-3807.

Demmig-Adams B., Adams W.W., Barker D.H., Logan B.A., Bowling D.R. and Verhoeven A.S. 1996 Using chlorophyll fluorescence to assess the fraction of absorbed

light allocated to thermal dissipation of excess excitation. Physiologia Plantarum 98, 253-264.

Eberhard S., Finazzi G. and Wollman F.A. 2008 The dynamics of photosynthesis. Annu. Rev. Genet. 42, 463-515.

Falkowski P.G. and LaRoche J. 1991 Acclimation to spectral irradiance in algae. J. Phycol. 27, 8-14.

Falkowski P.G. and Owens T.G. 1980 Light-Shade Adaptation : two strategies in marine phytoplankton. Plant Physiol 66, 592-595.

Fisher T., Berner T., Iluz D. and Dubinsky Z. 1998 The kinetics of the photoacclimation response of Nannochloropsis sp. (Eustigmatophyceae): A study of changes in ultrastructure and PSU density. Journal of Phycology 34, 818-824.

Fisher T., Minnaard J. and Dubinsky Z. 1996 Photoacclimation in the marine alga Nannochloropsis sp (Eustigmatophyte): A kinetic study. Journal of Plankton Research 18, 1797-1818.

Fisher T., Schurtz-Swirski R., Gepstein S. and Dubinsky Z. 1989 Changes in the levels of ribulose-1,5-biphosphate carboxylase / oxygenase (Rubisco) in Tetraedronminimum (Chlorophyta) during light and shade adaptation. Plant Cell Physiol. 30, 221-228.

Gouveia L. and Oliveira A.C. 2009 Microalgae as a raw material for biofuels production. J. Ind. Microbiol. Biotechnol. 36, 269-274.

Greenspan P., Mayer E.P. and Fowler S.D. 1985 Nile red: a selective fluorescent stain for intracellular lipid droplets. J. Cell Biol. 100, 965-973.

Guillard R.R.L. and Ryther J.H. 1962 Studies of marine planktonic diatoms. I. Cyclotella nana Hustedt and Detonula confervagea Cleve. Can. J. Microbiol. 8, 229-239.

Hodgson P.A., Henderson R.J., Sargent J.R. and Leftley J.W. 1991 Patterns of Variation in the Lipid Class and Fatty-Acid Composition of Nannochloropsis-Oculata (Eustigmatophyceae) During Batch Culture .1. the Growth-Cycle. Journal of Applied Phycology 3, 169-181.

Kim C., Meskauskiene R., Apel K. and Laloi C. 2008 No single way to understand singlet oxygen signalling in plants. EMBO Rep. 9, 435-439.

Laemmli U.K. 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Li Z., Wakao S., Fischer B.B. and Niyogi K.K. 2009 Sensing and responding to excess light. Annu. Rev. Plant Biol. 60, 239-260.

Maxwell K. and Johnson G.N. 2000 Chlorophyll fluorescence - a practical guide. J. Exp. Bot. 51, 659-668.

Moran R. and Porath D. 1980 Chlorophyll determination in intact tissues using n,n-dimethylformamide. Plant Physiol 65, 478-479.

Murata N., Takahashi S., Nishiyama Y. and Allakhverdiev S.I. 2007 Photoinhibition of photosystem II under environmental stress. Biochim. Biophys. Acta 1767, 414-421.

Nixon P.J., Michoux F., Yu J., Boehm M. and Komenda J. 2010 Recent advances in understanding the assembly and repair of photosystem II. Ann. Bot. 106, 1-16.

Packer A., Li Y., Andersen T., Hu Q., Kuang Y. and Sommerfeld M. 2011 Growth and neutral lipid synthesis in green microalgae: a mathematical model. Bioresour. Technol. 102, 111-117.

Peers G., Truong T.B., Ostendorf E., Busch A., Elrad D., Grossman A.R., Hippler M. and Niyogi K.K. 2009 An ancient light-harvesting protein is critical for the regulation of algal photosynthesis. Nature 462, 518-521.

Porra R.J., Thompson W.A. and Kriedemann P.E. 1989 Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b

extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim. Biophys. Acta 975, 384-394.

Rodolfi L., Chini Z.G., Bassi N., Padovani G., Biondi N., Bonini G. and Tredici M.R. 2009 Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol. Bioeng. 102, 100-112.

Sforza E., Bertucco A., Morosinotto T. and Giacometti G.M. 2010 Vegetal oil from microalgae: species selection and optimization. Chemical Engineering Transactions 2, 199-204.

Singh A., Nigam P.S. and Murphy J.D. 2011 Renewable fuels from algae: an answer to debatable land based fuels. Bioresour. Technol. 102, 10-16.

Solovchenko A., Khozin-Goldberg I., Recht L. and Boussiba S. 2010 Stress-Induced Changes in Optical Properties, Pigment and Fatty Acid Content of Nannochloropsis sp.: Implications for Non-destructive Assay of Total Fatty Acids. Mar. Biotechnol. (NY) in press.

Walters R.G. 2005 Towards an understanding of photosynthetic acclimation. J. Exp. Bot. 56, 435-447.

Wellburn A.R. 1994 The Spectral Determination of Chlorophyll-A and Chlorophhyll-B, As Well As Total Carotenoids, Using Various Solvents with Spectrophotometers of Different Resolution. Journal of Plant Physiology 144, 307-313.

Zou N. and Richmond A. 2000 Light-path length and population density in photoacclimation of Nannochloropsis sp (Eustigmatophyceae). Journal of Applied Phycology 12, 349-354.

CHAPTER 3

LIGHT INTENSITY AND CO_2 CONCENTRATION EFFECT ON *NANNOCHLOROPSIS GADITANA* GROWTH AND LIPIDS ACCUMULATION

Diana Simionato, Chiara Pistorello and Tomas Morosinotto contributed to this chapter

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3.1. INTRODUCTION

This brief chapter is a follow up of the previous one analyzing light influence on growth and lipids accumulation in *Nannochloropsis* cells exposed to a large range of illumination intensities. It was demonstrated that *Nannochloropsis gaditana* could be considered a good candidate for the outdoor biodiesel production because of its ability to adapt to very different light regimes that usually characterize the natural environment. In addition, it was found that light stress alone does not induce lipids accumulation at difference with other reports in the literature (Simionato et al., 2011).

However, in a large scale production perspective light influence should be considered together with other parameters influencing algae productivity. One major factor is carbon dioxide (CO_2) availability. As previously reported in (Guschina and Hartwood, 2006, Bhola et al., 2011) algae are able to exploit CO_2 externally provided to support and increase the biomass productivity. Also, CO_2 concentration has been suggested to influence both lipid content and composition in many algae (Guschina and Hartwood, 2006).

For this reason, in this chapter the work first presented in chapter 2 was repeated with a few distinct light intensities (low, control and high light) in the presence of excess CO_2 (5%) in order to verify the combined effect of light intensity and carbon dioxide on growth and lipids accumulation in *Nannochloropsis gaditana* cultures. Results showed that excess CO_2 strongly increased the growth rate at all light intensities. It has to be noted that the growth rate in the exponential phase was similar for the different light conditions, confirming *Nannochloropsis* ability to grow well under a wide range of illuminations. When lipids productivity was assessed, instead, the presence of excess

CO_2 determined a large difference in the final lipid amount in the different cultures showing that it was strongly enhanced under high light.

3.2. MATERIAL AND METHODS

3.2.1. Culture conditions

Nannochloropsis gaditana from CCAP, strain 849/5, was always grown in sterile filtered F/2 medium (Guillard and Ryther, 1962), using sea salts 32 g/l from SIGMA, 40 mM TRIS HCl pH 8, SIGMA Guillard's (F/2) marine water enrichment solution 1x. Maintenance and propagation of cultures were performed using the same medium added with 10 g/l of Plant Agar (Duchefa Biochemie). Growth experiments were performed in 0.25-L glass bubbled tubes agitated by air flowing with 5% CO_2 through a ceramic frit, starting from a pre-culture grown at 100 μE $m^{-2}s^{-1}$ at exponential phase which was diluted to an Optical Density (OD) value at 750 nm equal to 0.2. Illumination intensities were 15 and 100 μE $m^{-2}s^{-1}$ (daylight fluorescent lamps) and 1200 μE $m^{-2}s^{-1}$ (LED Light Source SL 3500, Photon Systems Instruments). Temperature was kept at 23 ± 1 °C in a growth chamber. Algal growth was measured by daily changes in optical density OD_{750} determined spectrophotometrically with a Lambda Bio 40 UV/VIS Spectrometer (Perkin Elmer) and cells number monitored with a Bürker Counting Chamber (HBG, Germany) under light microscope. In the logarithmic growth phase cells number was related to optical density. All curves were repeated at least three times and a culture grown at 100 μE $m^{-2}s^{-1}$ at atmospheric CO_2 was always present as a control.

3.2.2. In vivo monitoring of photosystem II quantum yield

Chlorophyll fluorescence was determined in vivo using Dual PAM 100 from WALZ. The parameter Fv/Fm was calculated as (Fm-Fo)/Fm (Demmig-Adams et al., 1996) after 20 minutes of dark adaptation.

3.2.3. Pigments extraction and analysis

Chlorophyll a and total Car were extracted from *Nannochloropsis* centrifuged cells at 4°C with 100 % N,N'-dimethylformamide for at least 48 hours in dark conditions as in (Moran and Porath, 1980). The pigments concentrations were determined spectrophotometrically using specific extinction coefficients (Porra et al., 1989; Wellburn, 1994).

3.2.4. Lipids analysis

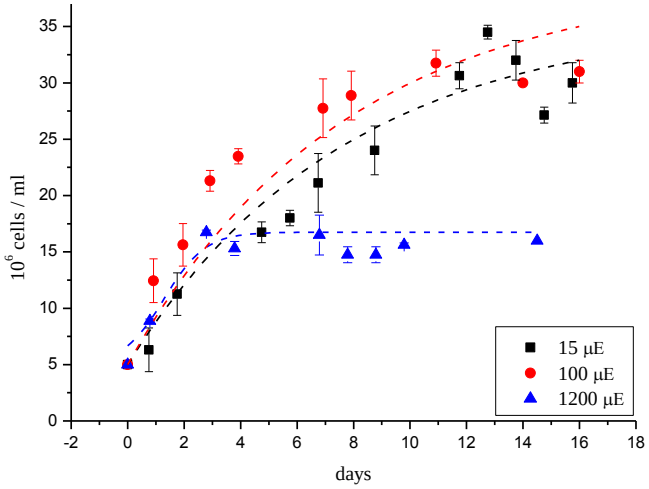
The lipids content was determined by staining 2 millions of algal cells centrifuged and re-suspended in 1.9 ml of deionized sterile water with Nile Red (NR) dye at final concentration of 2.5 µg/mL, for 10 minutes at 37°C (Chen et al., 2009). The fluorescence was measured using a spectrofluorometer (OLIS DM45), with excitation wavelength at 488 nm and emission wavelength in the range of 500 and 700 nm (Greenspan et al., 1985). The relative fluorescence of Nile Red for the lipids was obtained after subtraction of the auto-fluorescence of algal cells and Nile Red alone.

3.3. RESULTS

3.3.1. *Nannochloropsis* growth under different light intensities and 5% CO_2

Nannochloropsis gaditana was grown in 0.25-L glass bottles bubbled with air with 5% CO_2 . Cultures were grown under very different light conditions, respectively 15 (Low Light, LL), 100 (Control Light, CL), and 1200 (High Light, HL) µE $m^{-2}s^{-1}$. In all cases,

the growth kinetic was followed by monitoring the increase in cells number, as shown in figure 1. Excess CO_2 cultures exposed to 15 and 100 µE $m^{-2}s^{-1}$ showed a similar growth kinetic while high light cultures reached earlier their stationary phase and showed 50% less cells respect with LL and CL cultures. However, as reported in figure 1, growth rates calculated in the first days of exponential phase were very similar for the three light intensities tested. When growth rates are compared with the cells grown with the same light intensity and atmospheric CO_2 (chapter 2, figure 1) it is clear that cells use this external supply to increase their growing speed (figure 1). However, the maximal final cells number was the same between the two CO_2 conditions, suggesting other factors are limiting the growth in these conditions.



Light intensity (µE m^{-2} s^{-1})	Growth rate μ (day^{-1})
15	0.45 ± 0.08
100	0.46 ± 0.11
1200	0.41 ± 0.09

Figure 1. *Nannochloropsis gaditana* growth curve under different light regimes and 5 % CO2. Cells number for culture grown at 15, 100 and 1200 µE (black squares, red circles and blue triangles respectively). Fitting the experimental points are also shown as dashed lines. Box in the right shows the specific growth rate (µ) calculated for all growth curves. Calculations were done for the first days of exponential phase.

3.3.2. Photosystem II efficiency

In order to understand how the combination of light intensity and 5% CO_2 could influence the photosynthetic activity, the Photosystem II quantum yield was monitored during the entire growth through the exploitation of the fluorescence parameter Fv/Fm, which allows to detect the presence of photo-induced damage to PSII known to be the major target of photoinhibition (Murata et al., 2007). This parameter conserved a similar trend for all light intensities (figure 2). In fact Fv/Fm values were higher during the exponential phase and decreased steadily during stationary phase. While in LL cultures such a decrease is barely detectable, CL and HL cultures registered a strong decline in the PSII efficiency. This decrease was faster with the strongest light intensity suggesting a strong photo-sensitivity of these cells. The same trend was also observed with cells growing with atmospheric CO_2 cells (chapter 2, figure 2).

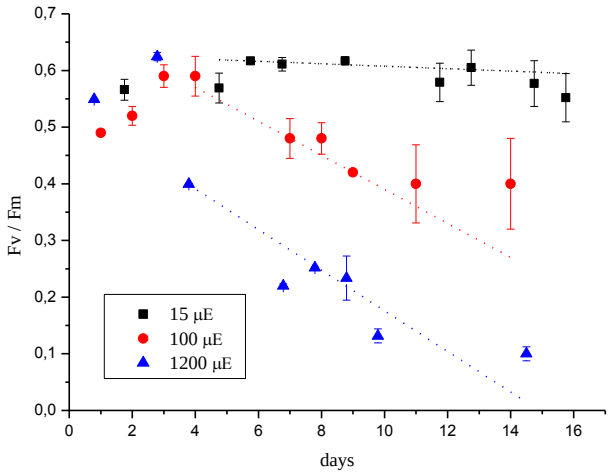


Figure 2. Photosynthetic parameters in *Nannochloropsis* cells acclimated to different light conditions and 5 % CO2. During growth curves reported in figure 1 Photosystem II efficiency was also monitored by determining the fluorescence parameter Fv/Fm. Here data for 15, 100 and 1200 µE (black squares, red circles and blue triangles respectively) are reported. The tendency during stationary phase is underlined by a dashed line.

3.3.3. Pigments analyses

One of the first adaptive response to variable light conditions in algae is the modulation of pigments contents. As in the previous conditions tested for *Nannochloropsis* (chapter 2, table 1), even in the presence of excess CO_2 in this work chlorophyll content in *N. gaditana* was strongly influenced by the light regime. Chl content per cell in HL cells after 3 days of the growth was ~4 times lower than LL cells (table 1). HL also stimulated an increase in Car/Chl ratio as reported in table 1. Modulation of pigment content thus suggested the activation of an acclimation response.

Table 1. Pigments content in *Nannochloropsis* cells grown at 15, 100 and 1200 µE and 5% CO_2. Chl content per cell and Car/Chl (µg/µg) ratio in cultures grown in different illumination regimes at day 3 are reported.

Illumination Intensity ($\mu E\ m^{-2}\ s^{-1}$)	**µg Chl / 10^6 cell**	**Car /Chl**
15	0.225 ± 0.012	0.22 ± 0.02
100	0.10 ± 0.09	0.35 ± 0.015
1200	0.06 ± 0.004	0.44 ± 0.015

3.3.4. Nannochloropsis lipids accumulation

Nannochloropsis is able to accumulate large amounts of lipids (Gouveia and Oliveira, 2008, Sukenik et al., 2009; Sforza et al., 2010) especially when exposed to a nutrient limited environment. In the previous chapter it was demonstrated how light alone cannot induce lipids accumulation when cells are grown at atmospheric CO_2 concentration. Here it was evaluated if the exposure to different illumination intensities combined with excess CO_2 caused different effects (figure 3). During the first nutrient rich phase of the growth LL and CL showed no lipid accumulation over the constitutive

cellular content of membranes. On the contrary, HL culture seemed to show since the very first days an increase in lipids content. During the stationary phase, lipids started accumulating also at the lowest intensities but even in this phase a higher illumination induced a stronger accumulation of this compounds. This is different from what observed in the previous chapter which showed how excess light did not produce any particular response in this sense (chapter 2, figure 6).

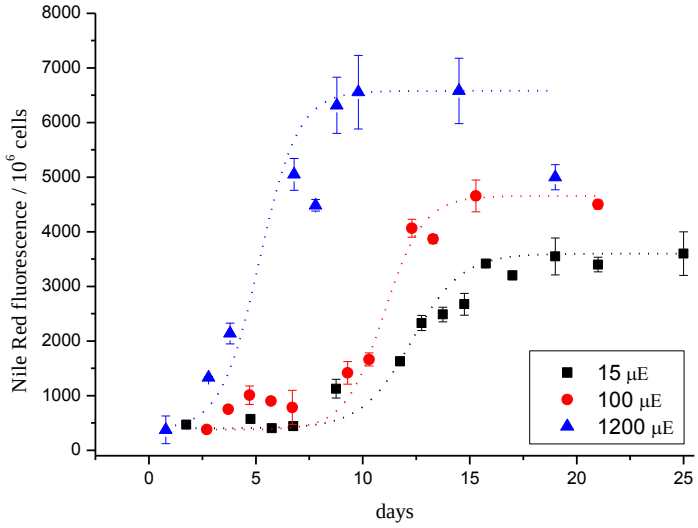


Figure 3. Lipids accumulation under different illumination. Lipids were quantified from the fluorescence at 585 nm of Nile Red stained cells.

3.4. DISCUSSION

3.4.1. Nannochloropsis exploits CO_2 for the growth during the exponential phase

This work aims to integrate the previous chapter including excess CO_2 (5%) in the analysis of the microalga *N. gaditana* response to different illumination intensities. Results first of all showed that in excess CO_2 growth is enhanced, as expected for a microalga. In fact in the tested conditions cells duplication time was half than with atmospheric CO_2 levels. Results still confirmed that at least during the nutrient rich

exponential phase *Nannochloropsis* has the remarkable ability of maintaining the same growth rate at every light conditions tested (figure 1). As expected, the pigment content modulated its composition, registering increased chlorophyll per cell in LL and increased carotenoids in HL. These are expected signs of the activation on an acclimation response, as observed in the previous chapter (chapter 2, table 1).

3.4.2. Light affects photosynthesis efficiency

During the first phase of the growth, *Nannochloropsis* was able to support the photochemistry reactions and maintained a good photosynthetic efficiency also under strong illumination. However, when cells reached the nutrient limited stationary phase, the analyses of the PSII efficiency (figure 2) showed how cells are more prone to photoinhibition. This is probably due to the rapid turnover of proteins of PSII complex in illuminated cells (Nixon et al., 2010). When nutrients are exhausted and protein synthesis is affected, there is a reduction in PSII repair which is reflected in a decreased Fv/Fm. In cultures grown with atmospheric (chapter 2, figure 2) and excess CO_2 (figure 2) appears the same trend, confirming this was mainly due to the total amount of light reaching the cells. However, previous works demonstrated that *Nannochloropsis* cells under nutrients deprivation were still able to increase the biomass (Sforza et al. 2010) probably due to lipids accumulation, confirming that in nutrients limitation *Nannochloropsis* is able to maintain a residual ability to exploit photosynthesis not for cells duplication but for support lipids biosynthesis.

3.4.3. *Nannochloropsis* exploits CO_2 under high light for an enhanced lipid accumulation

During the nutrient limited phase of the curve for all light intensities was verified an increased amount of lipids and this is in accord with previous works in which nutrients

deprivation was responsible for the lipids accumulation in some algae (Gouveia et Oliveira, 2008, Rodolfi et al., 2009). In this work when high light (1200 μE $m^{-2}s^{-1}$) and excess CO_2 were combined lipids accumulation was the highest. High light in atmospheric CO_2, on the contrary, induced a similar level of stress to the cells, judging by the decrease in Fv/Fm values, but this did not implied an increased lipids accumulation (chapter 2, figure 6). Results presented thus allowed concluding that under excess CO_2 and HL there likely was an over-accumulation of reduced carbon molecules which induced an enhanced lipids accumulation. Concluding, CO_2 availability could be considered a major factor for growth rate as well as for the maximal lipid productivity in *Nannochloropsis* cells.

3.5. REFERENCE LIST

Bhola V., Desikan R., Santosh S. K., Karthikeyan S., Sanniyasi E. and Bux F. (2011). Effects of parameters affecting biomass yield and thermal behaviour of Chlorella vulgaris. Journal of Bioscience and Bioengineering.

Chen W., Zhang C., Song L., Sommerfeld M. and Hu Q. (2009). A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. Journal of Microbiological Methods.

Demmig-Adams B., Adams W. W., Barker D. H., Logan B. A., Bowling D. R. and Verhoeven A. S. (1996). Using chlorophyll fluorescence to assess the fraction of absorbed light allocated to thermal dissipation of excess excitation. Physiologia Plantarum.

Gouveia L. and Oliveira A. C. (2009). Microalgae as a raw material for biofuels production. Journal of Industrial Microbiology and Biotechnology.

Greenspan P., Mayer E. P. and Fowler S. D. (1985). Nile red: a selective fluorescent stain for intracellular lipid droplets. Journal of Cell Biology.

Guillard R. R. L. and Ryther J. H. (1962). Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervagea* Cleve. Canadian Journal of Microbiology.

Guschina I. A. and Hartwood J. L. (2006). Lipids and lipid metabolism in eukaryotic algae. Progress in Lipid Research.

Moran R. and Porath D. (1980). Chlorophyll determination in intact tissues using n,n-dimethylformamide. Plant Physiology.

Murata N., Takahashi S., Nishiyama Y. and Allakhverdiev S.I. (2007). Photoinhibition of photosystem II under environmental stress. Biochimica et Biophysica Acta.

Nixon P.J., Michoux F., Yu J., Boehm M. and Komenda J. (2010). Recent advances in understanding the assembly and repair of photosystem II. Annuals of Botany.

Morosinotto T., Bonente G., Govoni C., Bassi R. (2009). Biocombustibili da alghe unicellulari. Le piante industriali per la multifunzionalità e sostenibilità dell'agricoltura italiana nel terzo millennio. Edizioni Ranalli.

Porra R. J., Thompson W. A. and Kriedemann P. E. (1989). Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochimica et Biophysica Acta.

Rodolfi L., Chini Z. G., Bassi N., Padovani G., Biondi N., Bonini G. and Tredici M. R. (2009). Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnology and Bioengineering.

Sforza E., Bertucco A., Morosinotto T. and Giacometti G. M. (2010). Vegetal oil from microalgae: species selection and optimization. Chemical Engineering Transactions.

Simionato D., Sforza E., Corteggiani Carpinelli E., Bertucco A., Giacometti G. M. and Morosinotto T. (2011). Acclimation of *Nannochloropsis gaditana* to different illumination regimes: effects on lipids accumulation. Bioresource Technology.

Sukenik A., Beardall J., Kromkamp J. C., Kopeck J., Masojídek J., Bergeijk S., Gabai S., Shaham E. and Yamshon A. (2009). Photosynthetic performance of outdoor *Nannochloropsis* mass cultures under a wide range of environmental conditions. Aquatic Microbial Ecology.

Wellburn A. R. (1994). The Spectral Determination of Chlorophyll-A and Chlorophhyll-B, As Well As Total Carotenoids, Using Various Solvents with Spectrophotometers of Different Resolution. Journal of Plant Physiology.

CHAPTER 4

EFFECTS OF NITROGEN AVAILABILITY ON *NANNOCHLOROPSIS GADITANA* PHOTOSYNTHETIC APPARATUS

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4.1. ABSTRACT

Algae have a promising potential as feedstock for biodiesel production but a strong research effort is still needed to fulfill their potential in large scale production and at least partially replace fossil fuels. Species of the genus *Nannochloropsis* are considered among the most promising ones for biodiesel production because of their ability to accumulate large amounts of lipids, especially when exposed to nutrients limitation, nitrogen in particular. Nitrogen is a key nutrient for any living organism, being fundamental for the synthesis of most biological molecules and the manipulation of its availability has several influences on algae physiology. In this work *Nannochloropsis gaditana* cells under nitrogen excess and deprivation conditions were characterized with particular attention on the effects on the photosynthetic apparatus and efficiency. Results showed that nitrogen deprivation induces a decrease of all photosynthetic components such as Photosystem II, Photosystem I and Cytochrome b_6f complex, with the former particularly affected. *Nannochloropsis*, however, is able to cope with nitrogen deprivation through a re-organization of the entire photosynthesis chain, altering the stoichiometry of PSII / PSI and increasing the exploitation of alternative electron pathways such as cyclic electron transport. Presented results suggest a picture where *Nannochloropsis* under nitrogen deprivation decreases the content in photosynthetic apparatus components while maintaining a good efficiency for the residual proteins.

4.2. INTRODUCTION

Microalgae are receiving a strong attention as possible feedstock for biofuels production (Chisti, Y. and Yan, J. Y. 2011, Malcata, F. X. 2011) and in particular for biodiesel

produced from lipid reserves. In fact, some species of microalgae are able to accumulate large amounts of these compounds which can reach concentrations up to 65-70% of total dry weight (Boussiba, S. et al. 1987, Hodgson, P. A. et al. 1991, Rodolfi, L. et al. 2009) with a potential productivity ten times higher than plants (Chisti, Y. 2008). In many algal species it has been observed that a major signal inducing lipids accumulation is nutrients limitation, with nitrogen deprivation particularly effective (Eltgroth, M. L. et al. 2005, Richardson, B. et al. 1969, Zhekisheva, M. et al. 2002). Nitrogen, however, is a major component of most biological molecules and its deprivation affects many aspects of the algae physiology besides lipids accumulation and its impact must be carefully assessed. Nutrients limitation is often experienced by algae in natural ecosystems, especially marines, where nutrients limitation affects photosynthetic efficiency and consequently biomass productivity (Berges, J. A. et al. 1996, Grossman, A. 2000, Kolber, Z. et al. 1988, Miller, R. et al. 2010). In order to respond to variability of natural environments algae show an extreme versatility in response to different nutrient supplies (see review by (Cardol, P. et al. 2011)).

In order to identify strategies for optimization of biomass productivity it is seminal to understand how the species of interest respond to environmental modifications and, in this case, nitrogen deprivation. Since one of the main limiting factors for large scale cultivation is light availability, algae to be effectively productive must be able to maintain photosynthetic efficiency and the ability to convert sunlight energy into biomass. In order to evaluate the possibility of exploiting nitrogen deprivation to produce lipids it is thus fundamental to assess in detail its effects on photosynthetic efficiency and eventually explore the possibility of generating transgenic algae with improved performances under these conditions.

Previous work showed that nitrogen deprivation inhibited photosynthesis in the green alga *Chlamydomonas* (Miller, R. et al. 2010), with Photosystem II particularly affected in both diatoms and green algae (Berges, J. A. et al. 1996). On the contrary, Photosystem I seemed to be more resistant to nitrogen deprivation, while being more sensitive to the absence of other nutrients such as iron (Berges, J. A. et al. 1996, Greene, R. M. et al. 1991). Nutrients availability has been also shown to lead to an inhibition of Linear Electron Flow (LEF) from PSII to PSI with the activation of alternative electron pathways such as chlororespiration and Cyclic Electron Flow (CEF) around PSI (Cardol, P. et al. 2011).

Species belonging to the genus *Nannochloropsis* are considered as some of the most promising candidates for the biodiesel production, thanks to their fast growth rate and ability to accumulate large amount of lipids (Gouveia, L. and Oliveira, A. C. 2009, Sukenik, A. et al. 2009). *Nannochloropsis* genus belongs to the Eustigmathophyceae and is composed mainly by species living in a coastal environment. They have cells of reduced size (3-5 μm) and present a single chloroplast occupying most of the cell. They also have a peculiar pigments content, presenting only chlorophyll (Chl) *a* and lacking other accessory chlorophylls such as Chl *b* or *c*. Violaxanthin and vaucheraxanthin are the most represented carotenoids in the cells (Lubian, L. M. et al. 2000). In the case of *Nannochloropsis* it has been shown that this oleaginous alga, as most other algae, responds to abiotic stresses by modulating its pigments content (Simionato, D. et al. 2011).

In this work *Nannochloropsis gaditana* response to nitrogen deficiency was investigated in detail, in order to clarify the mechanisms which allow a residual growth of the cells growing even when any source of additional nitrogen is provided to the culture. The main effort was focused on describing the photosynthetic apparatus response to this

condition inducing lipids accumulation. Results presented show that *Nannochloropsis* responds to nitrogen deprivation by re-organizing the entire photosynthesis chain, reducing the content in PSII, PSI and Cyt b_6f complex and increasing the activity of alternative electron pathways such as cyclic electron flow.

4.3. MATERIALS AND METHODS

4.3.1. Culture conditions

Nannochloropsis gaditana from CCAP, strain 849/5, was always grown in sterile filtered F/2 medium (Guillard, R. R. L. and Ryther, J. H. 1962), using sea salts 32 g/l from SIGMA, 40 mM TRIS HCl pH 8, SIGMA Guillard's (F/2) marine water enrichment solution 1x modified by adding non limiting nitrogen concentration ($NaNO_3$ 1.5g/L). Maintenance and propagation of cultures were performed using the same medium added with 10 g/l of Plant Agar (Duchefa Biochemie). Growth experiments were performed in Erlenmeyer flasks with magnetic agitation, starting from a pre-culture grown at 100 μE $m^{-2}s^{-1}$ at exponential phase which was diluted to an Optical Density (OD) value at 750 nm equal to 0.4, corresponding to ~$17*10^6$ cells/ml, final volume 150 ml. Algal growth was maintained for 4.5 days (pre-inoculum) and then cells were centrifuged at 2500g (Allegra 250, Beckman) for 10 minutes at RT and re-suspended in two different F/2 media, one presenting excess nitrogen ($NaNO_3$ 1.5 g/L), the other completely deprived of any nitrogen source and re-diluted to OD_{750} = 0.4. The growth was then performed for other 5 days. Illumination was constantly provided at 100 μE $m^{-2}s^{-1}$, using daylight fluorescent lamps. Temperature was kept at 23 ± 1 °C in a growth chamber. Algal growth was measured spectrophotometrically by daily changes in OD at 750 nm (Lambda Bio 40 UV/VIS Spectrometer, Perkin Elmer) and cells

number monitored with a Bürker Counting Chamber (HBG, Germany) under light microscope. All curves were repeated at least four times.

4.3.2. Pigments extraction and analyses

Chlorophyll *a* and total carotenoids were extracted from *Nannochloropsis* centrifuged cells at 4°C with 100% N,N'-dimethylformamide for at least 48 hours in dark conditions as in (Moran, R. and Porath, D. 1980). The pigments concentration were determined spectrophotometrically using specific extinction coefficients (Porra, R. J. et al. 1989, Wellburn, A. R. 1994).

HPLC analyses were carried out using a Beckman System Gold instrument equipped with an UV-VIS rilevator and a 18-C column (Zorbax Ods, 25cm x 4.6 mm). Cells were mechanically broken in a mortar using liquid nitrogen and quartz, pigments were extracted using acetone 80% and after centrifugation 200 µl of sample were loaded. Runs were performed as in (Farber, A. and Jahns, P. 1998) using acetonitrile 86.7%, methanol 9.6% and Tris/HCl pH 7.8 3.6% as solvent A and methanol 80%, hexane 20% as solvent B. The peaks of each sample were identified through the retention time and absorption spectrum and each chromatogram was normalized on the Chl *a* peak to compare carotenoids. For the picomolar quantification of carotenoids correction factors as in were used (Farber, A. and Jahns, P. 1998).

4.3.3. Western Blot analyses on proteins from isolated thylakoids

Isolation of thylakoid membranes was performed according to (Lepetit, B. et al. 2007) with some modifications. Cells from selected phase (before and after the re-suspension in the two nitrogen conditions) were harvested by centrifugation (Allegra 250, Beckman) at 4000 g for 5 minutes and then re-suspended in a variable volume of 0.4 M

NaCl, 2 mM $MgCl_2$, 20 mM Tricine/KOH pH 7.8 (B1 buffer). All following steps were performed at 4°C under dim light. Cells were centrifuged at 4000 g for 5 minutes and concentrated to an OD_{750} value equal to 50 with B1 added with 0.5% milk powder and protease inhibitors (1 mM PMSF, 1 mM DNP-ε-amino-*n*-caproic acid and 1 mM benzamidine). The cells were disrupted with French Press at pressure of 1.7 kBar for one cycle. Unbroken cells were separated from the broken ones by a centrifugation step at 2500g for 10 minutes, then re-suspended in B1 with 0.5% of milk powder and inhibitors as previously described, processed once through French Press and centrifuged as before. Broken cells of the two previous steps were mixed and subjected to a new French Press cycle at 1.7 kBar and centrifuged at 15000g for 20 min (5417R, Eppendorf). The pellet was re-suspended in a variable volume of 0.15 M NaCl, 5 mM $MgCl_2$, 20 mM Tricine/KOH pH 7.8 (B2 buffer) and centrifuged at 15000g for 20 min. This step was repeated twice. Finally, the pellet composed of thylakoids was re-suspended in a small amount of 0.4 M sorbitol, 15 mM NaCl, 5 mM $MgCl_2$, 10 mM Hepes/KOH pH 7.5 (B4 buffer) and immediately freezed in liquid nitrogen and stored at -80°C. Chl *a* was extracted with 80% acetone and the concentration was determined spectrophotometrically using specific extinction coefficients (Porra, R. J. et al. 1989, Wellburn, A. R. 1994) and the fitting as in (Croce, R. et al. 2002). For the evaluation of D1, PsaA and Cyt b_6f content, solubilized thylakoids were loaded using 6 µg Chl *a*. The solubilization buffer was 30% glycerol, 125 mM Tris pH 6.8, 0.1 M DTT, 9% SDS (SB3X). SDS-PAGE analysis was performed with a Tris–Glycine buffer system as in (Laemmli, U. K. 1970) with 12 % acrylamide. Western Blot analyses were performed using nitrocellulose (Pall Corporation) afterward incubated with specific antibodies.

4.3.4. In vivo monitoring of photosystem II quantum yeld

Chlorophyll fluorescence was determined in vivo using Dual PAM 100 from WALZ. The parameters Fv/Fm was calculated as (Fm-Fo)/Fm (Demmig-Adams, B. et al. 1996) after 20 minutes of dark adaptation.

4.3.5. Spectroscopic analyses on photosynthetic components: Electrochromic Shift

The Electrochromic Shift (ECS) spectral change is correlated to the number of light-induced charge separations within the reaction centers. Thus, the PSII contribution can be calculated from the decrease in the signal amplitude upon the addition of DCMU and HA which respectively inhibits PSII and prevents the recombination charge between $P680^+$ and Q_a^- acting as reducing agent once the sample has been pre-illuminated. PSI was estimated as the fraction of the signal that was insensitive to these inhibitors (Cardol, P. et al. 2009). To perform this analysis, we first assessed the in vivo spectrum of the ECS in *Nannochloropsis gaditana* in the 480-550 nm range upon continuous illumination of 1000 µE $m^{-2}s^{-1}$ as discussed earlier (Bailleul, B. et al. 2010). PSI and PSII content was estimated spectroscopically from changes in the amplitude of the fast phase of the ECS signal (at 527 nm and 507 nm, where the positive and negative peaks of the signal are respectively located) upon excitation with a saturating laser flash (520 nm, 5 ns duration). PSII contribution was calculated from the decrease in the signal amplitude upon addition of DCMU (80 µM) and hydroxylamine (4 mM) that irreversibly block PSII charge separation. Conversely, PSI was estimated as the fraction of the signal that was insensitive to these inhibitors. Cytochrome f, b_6 and P700 were estimated by evaluating the maximum absorption change in samples incubated with DBMIB. This compound blocks plastoquinol oxidation by the cytochrome b_6f complex, thus leading to a full oxidation of both PSI and cytochrome redox cofactors. Cyt f and

b_6 were respectively evaluated by subtracting absorption at 554 nm and 563 nm from a baseline drawn between 546 and 573 nm . P700 was measured at 705 nm. Measurements of linear, cyclic and other alternative electron flows were also deduced by measuring PSI turnover in untreated, DCMU poisoned (to block linear flow) and DCMU and DBMIB treated samples (to block both linear and cyclic flows). CEF was then evaluated as the residual rate of $P700^+$ reduction that was observed in the presence of DCMU, but abolished by DBMIB addition. All spectroscopic measurements were performed using a JTS-10 (BioLogic, France).

4.4. RESULTS

4.4.1. Effects of nitrogen excess or deprivation on *Nannochloropsis* growth

Nannochloropsis gaditana was grown in batch cultures under a continuous dim light of 100 μE $m^{-2}s^{-1}$. In order to ensure the standardization of the pre-inoculum, cells were first grown for 4.5 days in a F/2 medium replete for nitrogen. Before reaching the stationary phase cells were harvested and used to start two different cultures, one with the same fresh medium and another one completely lacking nitrogen. Growth kinetics, reported in figure 1, showed that *Nannochloropsis* has the remarkable ability to maintain a good and similar growth rate during the 5 days after the re-suspension in the two media even in the absence of any additional nitrogen source.

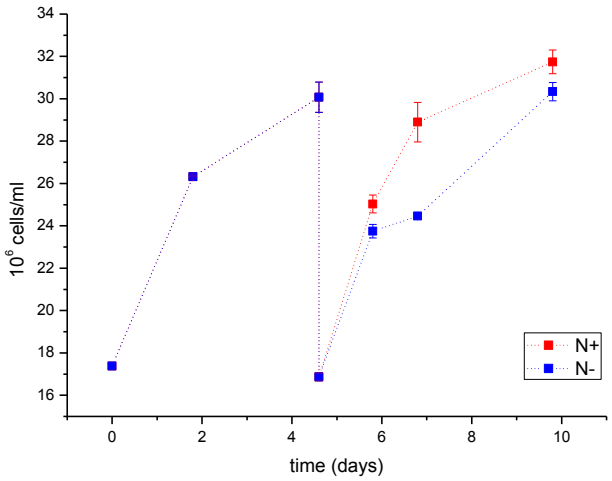


Figure 1. **Growth of *Nannochloropsis gaditana* cultures with Nitrogen excess or Nitrogen deprivation**. Cells were grown 4.5 days in a nitrogen rich medium, then harvested and split into two other media: one N rich (red) and the other completely lacking of any additional N source (blue).

4.4.2. Photosynthetic acclimation to different N conditions: modulation of pigments and protein content

First of all, in both nitrogen conditions the pigment content was evaluated, whose changes are indicative of a modulation of photosynthetic apparatus composition. As shown in table 1, N availability has a large influence on Chl content per cell which in N- cells is around 50% of control and N+ cells, which are indistinguishable. Also Chl/Car ratio decreased in N- cells (table 1), suggesting a relative increase in total carotenoids content. When individual pigment species were analyzed by HPLC (Table 2), we observed that N- cells showed a specific relative increase in violaxanthin, anteraxanthin and zeaxanthin, the carotenoids participating to the xanthophylls cycle which is a well-known mechanism of photoprotection (Demmig-Adams, B. and Adams, W. W. 2000, Niyogi, K. K. et al. 1997). We also observed enhanced accumulation of a new carotenoid species, identified as the secondary carotenoid cantaxanthin.

Table 1. Pigment composition of N excess or N deprived cells. Chl content per 10^6 cells and Chl/car (μg/μg) ratio in cultures before (pre-inoculum) and after the resuspension in the two N conditions (excess and deprivation) are reported.

Nitrogen condition	μg chl a / 10^6 cells	Chl/car
Pre-inoculum	0.147 ± 0.011	2.84 ± 0.098
N+	0.146 ± 0.023	2.86 ± 0.014
N-	0.067 ± 0.032	2.54 ± 0.021

Table 2. Carotenoids composition of *N. gaditana* cells after the re-suspension in N excess or N deprived conditions. The number of carotenoid moles over 100 Chl a moles in the two N conditions from HPLC analyses are reported.

Nitrogen condition	Viola	Vauchera	Antera	Vauchera ester	Zea	Canta	β- car
Pre-inoculum	30±0.56	10.2± 0.02	4.3 ± 0.2	3.5 ± 0.2	3.5±0.3	1.5±0.5	1.8±0.1
N+	27±0.14	9 ± 0.02	3.4 ± 0.2	3.5 ± 0.3	1.9±0.6	1.2±0.7	1.6±0.01
N-	50.2±1.5	12.7 ± 0.4	8.1 ± 0.5	3.6 ± 0.2	7.3±0.2	6.6±0.3	1.1±0.2

We next assessed if N deprivation affected specifically some of the complexes involved in photochemistry. D1 of Photosystem II and PsaA of Photosystem I content in *N. gaditana* cultures adapted to N excess and N deprivation was monitored using specific antibodies. Western blotting in figure 2A showed a clear decrease of D1 content in N deprived medium. On the contrary, PsaA showed a relative increase in N deprived cultures (figure 2B). It is worth mentioning that samples were loaded using equivalent Chl *a* amounts to ensure a sufficient signal level. Western Blot analyses on other proteins involved in the photochemistry such as Cyt b_6f complex showed a decrease in N- cells even in this case relatively to chlorophyll (figure 2C).

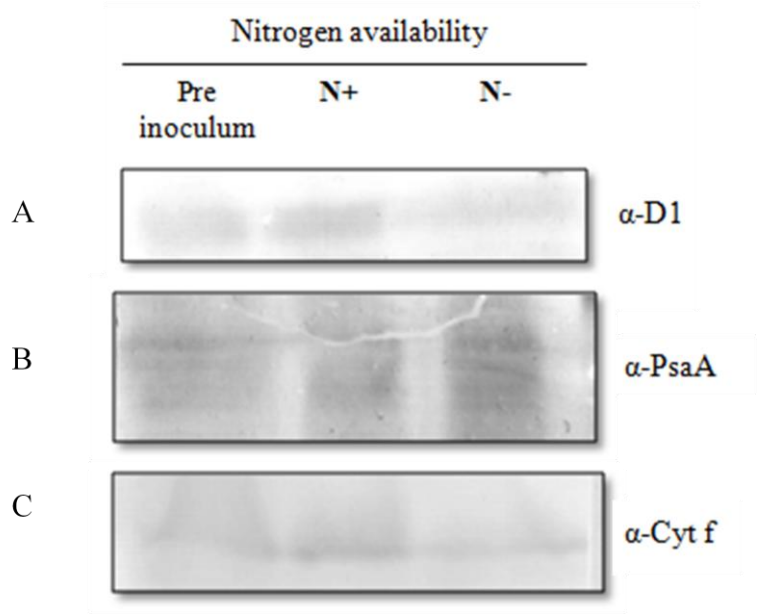


Figure 2. Photochemical efficiency in N excess and N deprived cells. Evaluation of A) D1, B) PsaA and C) Cyt f content, determined using specific antibodies. Each sample was loaded using the same chlorophyll *a* amount, 6 µg (thylakoids).

4.4.3. Photosynthetic apparatus composition in different N conditions

In order to better understand how *Nannochloropsis* photosynthetic apparatus responds to N excess and deprivation, in this work the Electrochromic Shift (ECS) was employed to assess possible changes in the PSII/PSI stoichiometry. ECS kinetics exploit the ability of pigments, and in particular carotenoids, to undergo a shift in the absorption spectrum during the exposure to continuous high light (Bailleul, B. et al. 2010). In order to apply this method, it was first measured the spectrum of the ECS signal in *Nannochloropsis gaditana*, owing to its peculiar pigments when compared to *viridiplantae*: in fact violaxanthin and vaucheraxanthin are together with Chl *a* the major photosynthetic pigments of this microalga (Brown, J. S. 1987, Lubian, L. M. et al. 2000) (Table 2). The overall *Nannochloropsis* cells ECS spectrum (figure 3) showed clear similarities with the others recorded in other algae (Bailleul, B. et al. 2010), although its positive peak was red shifted, probably reflecting the involvement of the

Nannochloropsis major carotenoids (violaxanthin and vaucheriaxanthin) in the signal. Consistent with this possibility, the width of the peak suggests that several carotenoids are contributing to the ECS signal.

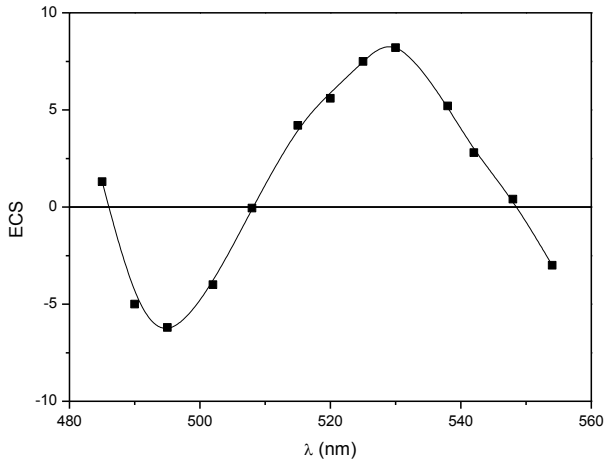
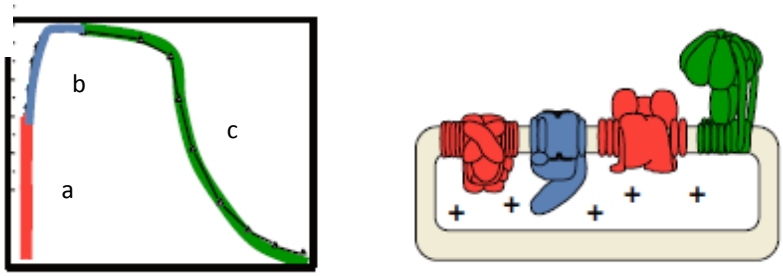


Figure 3. Electrocromic shift (ECS) signal of *Nannochloropsis gaditana*. Algae were illuminated with continuous light and the ECS spectrum is presented as the light minus the dark signal in the range between 480 and 550 nm.

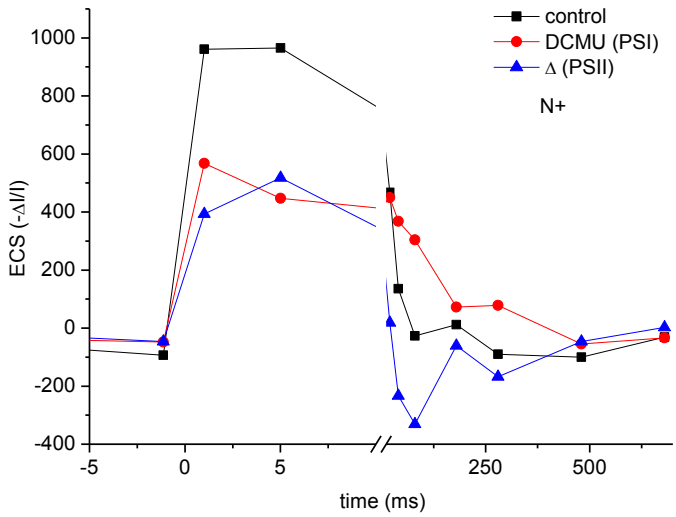
ECS spectroscopy identifies three phases in the kinetic (Joliot, P. and Delosme, R. 1974), providing information about the charge separation by the photosystems (first phase, a in figure 4A), Cyt b_6f complex activity (second phase, b in figure 4A) and ATP-synthase activity (third phase, c in figure 4A) (Bailleul, B. et al. 2010). By focusing on the signal maximum (527 nm) it was possible to quantify PSII/PSI ratio in the two different N conditions exploiting the signal during the first phase of the ECS kinetic (Bailleul, B. et al. 2010). Using inhibitors like DCMU and HA, which respectively inhibits PSII and prevents the recombination charge between $P680^+$ and Q_a^- acting as reducing agent (see also material and methods), it was afterwards possible to distinguish the PSII and PSI relative contribution to *Nannochloropsis* ECS signal, as shown in figures 4B and 4C. Measurements performed with N+ and N- cells evidenced a decrease in PSII abundance with respect to PSI in N- cells. Their different

contribution can be precisely quantified, showing that PSII/PSI ratio was 0.95 ± 0.12 for N+ cells and 0.56 ± 0.07 for N- cells. These results showed that PSII has to be considered the major target of nitrogen deprivation.

A



B



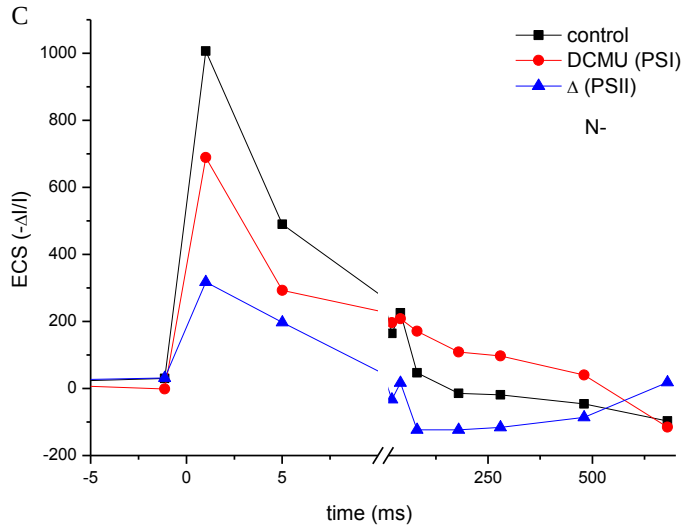
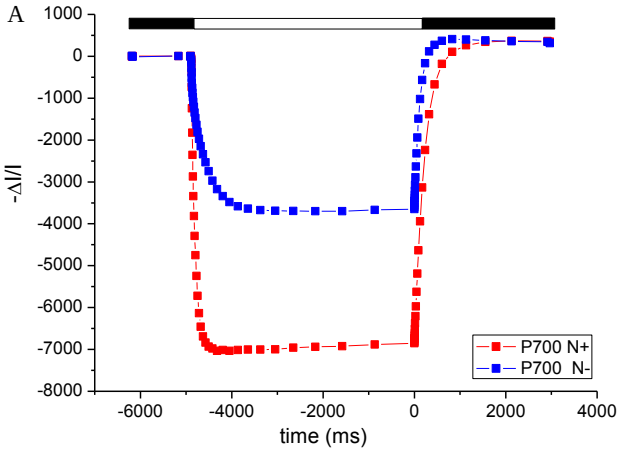


Figure 4. ECS kinetics measured in intact cells of *Nannochloropsis* during a saturating laser pulse excitation. A) ECS basics from (Bailleul et al., 2010): ‘‘a’’ phase (red) corresponds to PSI and PSII charge separations, which occur on an unresolved timescale. The ms time range rise phase (‘‘b’’ phase, blue) corresponds to electron flow in the cytochrome b chain of the cytochrome b6f. The ECS decay (‘‘c’’ phase, green) is due to charge leakage through the membrane, mainly H^+ via the ATP-synthase. B) ECS kinetics at 527 nm of *Nannochloropsis* N+ cells for evaluating PSII/PSI ratio. C) ECS kinetics at 527 nm of *Nannochloropsis* N- cells for evaluating PSII/PSI ratio. C) and D) Black squares = control kinetic, red circles = kinetic after the addition of DCMU and HA, blue triangles = kinetic after the addition of DCMU, HA and DBMIB.

Difference absorption signals can also be exploited for the *in vivo* quantification of some components of the photosynthesis chain like P700 and Cyt b_6f complex. P700 could be detected from a differential absorption signal around 700 nm (fig. 5A), using a light source specifically exciting this photosystem (Bailleul, B. et al. 2010). This measurement allowed an absolute quantification of PSI per cells, which showed a significant decrease in this photosystem in nitrogen deprived cells (fig. 5D). It appeared therefore that nitrogen starvation affects both PSI and PSII (figures 4B and 4C), but the latter is more severely decreased upon starvation. We also evaluated the amount of functional cytochrome b_6f complexes by absorption spectroscopy. In particular, the appearance of signals reflecting the oxidation of b (563 nm) and f (554 nm) – type hemes upon incubation with DBMIB (a specific inhibitor of cyt b_6f) allowed

quantifying the amount of photo-oxidable cytochrome complexes (figures 5B and 5C). Overall, these results showed an absolute decrease of both these components under nitrogen deprivation when compared to the same number of cells of both nitrogen conditions. In particular in N- cells there were ~50% of both signals with respect to N+ cells (fig. 5D). It is worth mentioning that data kinetics at 554 nm showed in N- cells not only an alteration in signal intensity but also in kinetics parameters and when light was switched off N- cells showed a faster signal relaxation with respect to N+ cells. A possible explanation is the presence of Cyt c_6 which showed a signal at this wavelength comparable with cyt f (Buchel, C. and Garab, G. 1995). Normally, Cyt c_6 accumulation is induced in some algae species like diatoms under copper limitation, replacing plastocyanin as electron carrier between Cyt b_6f complex and PSI (Crowley, P. B. et al. 2002, Inda, L. A. et al. 1999). Algae accumulating Cyt c_6 showed similar effects in 554 nm differential absorption supporting the hypothesis that this might be induced in these conditions.



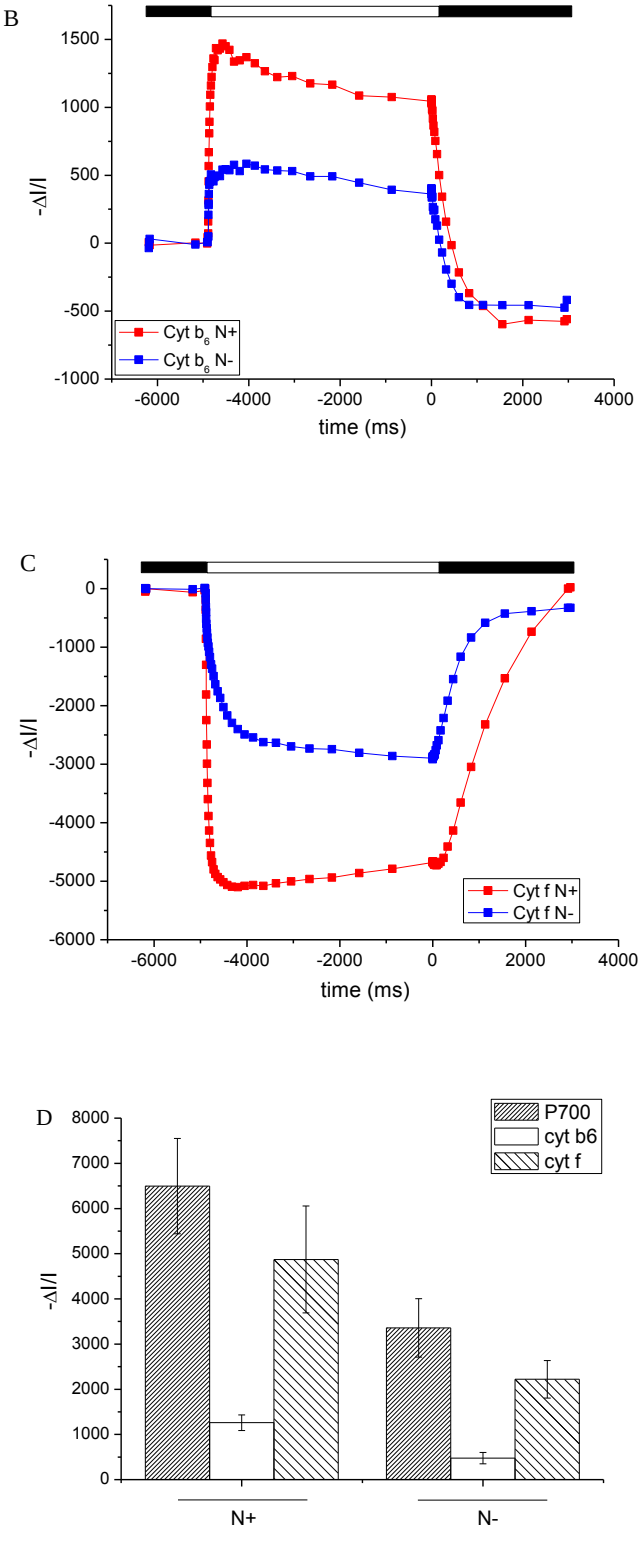


Figure 5. Differential absorption spectroscopy measured in intact cells of *Nannochloropsis* during a saturating laser pulse excitation. Data referred to the same cells number. Example for the detection of absolute content of A) P700 at 705 nm B) Cyt b_6 at 563 nm C) Cyt f at 554 nm. A), B) and C) red squares = N excess cells, Blue squares = N deprived cells, Black barrel = light switched off during the measurement, empty barrel = light switched on during the measurement. D) P700, Cit b_6 and Cyt f average values for all replicates.

4.4.4. Photosynthetic efficiency in different N conditions

All data presented showed a strong effect of nitrogen deprivation on *Nannochloropsis* photosynthetic apparatus, affecting all complexes of the electron transport chain. After assessing the composition of the photosynthetic apparatus it is important to analyze how nitrogen deprivation affects its activity. First it was compared the fluorescence parameter Fv/Fm, which allows monitoring the PSII quantum yield (Maxwell, K. and Johnson, G. N. 2000, Murata, N. et al. 2007) during growth. After the re-suspension in N excess or N deprived medium, cells showed a different behavior. In fact N+ cells continued to maintain a high Fv/Fm while N- cells showed a progressive decrease (figure 6). However, it must be noted that the reduction is not drastic and suggests that *Nannochloropsis* maintains a substantial photosynthetic efficiency.

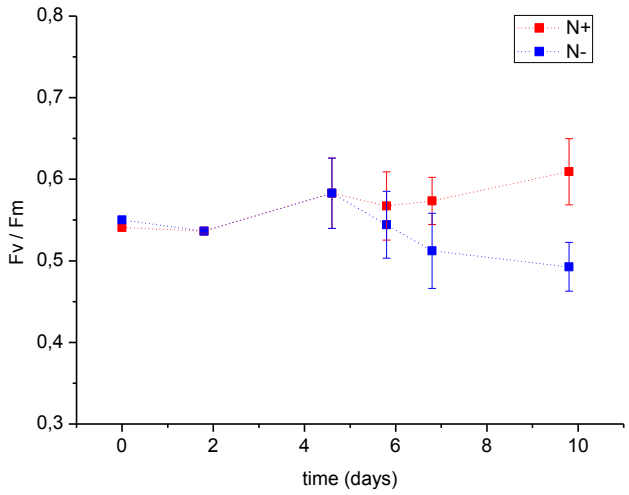


Figure 6. Photosynthetic parameters in *Nannochloropsis* cells acclimated to different N conditions. During growth curves reported in figure 1 Photosystem II efficiency was monitored by determining the fluorescence parameter Fv/Fm. Here data for N excess (red squares) and N deprived (blue squares) cultures.

In order to evaluate more deeply this hypothesis, electron transport efficiency was analyzed in more detail. Normally the main pathway for the electron transport along the photosynthesis chain is the Linear Electron Flow (LEF) from PSII to PSI and $NADP^+$,

but alternative pathways have been described in plants and algae (see review by (Peltier, G. et al. 2010)), such as Cyclic Electron Flow (CEF) around PSI (Peltier, G. et al. 2010), hydrogen production (Philipps, G. et al. 2011) and chlororespiration (Peltier, G. and Schmidt, G. W. 1991). Absorption spectroscopy at around 700 nm under continuous high illumination of 1000 μE $m^{-2}s^{-1}$ provided by a P700 light source is also useful to verify the total amount of electron flow in entire algal cells and the eventual balance between linear, cyclic and other electron flows (Bailleul, B. et al. 2010). As shown in figure 7 when comparing the same cells number of the two nitrogen conditions, *Nannochloropsis* showed that the overall electron flow rate was decreased under N deprivation. Furthermore, nitrogen depletion also affects electrons partitioning between the linear and cyclic pathways. This was evaluated using DCMU, which inhibits PSII, and DBMIB which blocks both linear and cyclic flows (see also material and methods) and results showed that linear electron flow was prominent in N+ cells while the relative contribution of cyclic flow significantly increased upon N deprivation. In addition to LEF and CEF there is also a residual component resistant to DCMU and DBIMIB presence which is attributable to other alternative electron flows. This component is stable in both conditions of N availability (figure 7), confirming that the increased CEF is the first applied strategy in *Nannochloropsis* to face off to nitrogen deprivation.

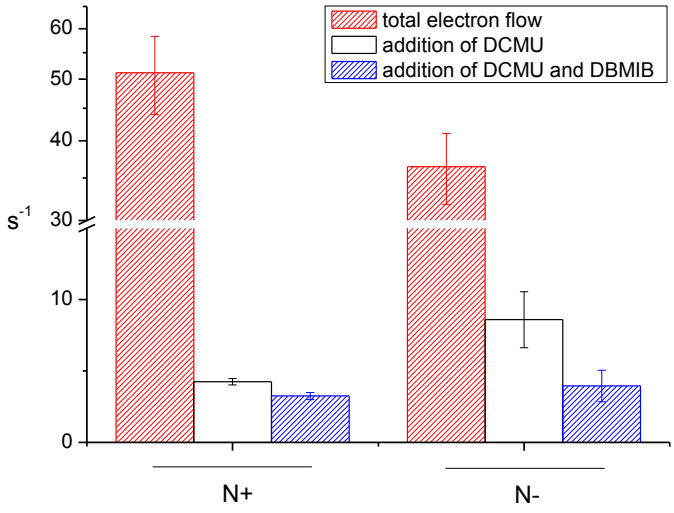


Figure 7. Spectroscopic quantification of the total and alternative electrons flow under N excess and N deprivation conditions in *Nannochloropsis* cells. This parameter has to be referred at the same cells number. The red column represents the total electron flow. The white column shows the contribution of cyclic and other alternative electron flows after the addition of DCMU. Red column minus white column represents the linear electron flow. The blue column refers to other alternative electron flows after the addition of DCMU and DBMIB which respectively block linear and cyclic electron flows. White column minus blue column represents the cyclic electron flow.

4.5. DISCUSSION

4.5.1. *Nannochloropsis gaditana* is able to grow under nitrogen deprivation

Nannochloropsis gaditana cultures were grown for 4.5 days in a F/2 medium replete for nitrogen and then harvested and used to start two different cultures, one with the same fresh medium and another one completely lacking nitrogen. Data reported in figure 1 showed that *Nannochloropsis* has a remarkable ability to maintain a good and similar growth rate during the 5 days after the re-suspension in the two media even in the absence of any additional nitrogen source. Since here *Nannochloropsis* is growing in full autotrophy, this result implies that cells maintained a good efficiency in converting light into biomass and thus that photosynthesis was efficient even in nitrogen deprivation. Clearly, residual nitrogen is still present from the pre-culture and cells are

capable of managing this residual amount to maintain a sufficient growth. For this reason, it is interesting to investigate which kind of response is activated in these conditions in *N. gaditana* and how the photosynthetic apparatus responds to nitrogen availability.

4.5.2. *Nannochloropsis* acclimates to nitrogen availability trough the re-organization of the entire photosynthetic apparatus

The change in the pigment content is indicative of a modulation of photosynthetic apparatus composition. The decrease of Chl per cell in N- cells verified in this work (Table 1) was expected for nutrients stressed cells since neither proteins nor the nitrogen-containing chlorophyll molecules can be synthesized upon starvation. On the contrary the total carotenoids content (on a chlorophyll basis) is increased (Table 1). Carotenoids are fundamental for protection from oxidative stress in photosynthetic organisms (Li, Z. et al. 2009) and their accumulation is often observed under stress conditions (Grunewald, K. et al. 1997, Lubian, L. M. et al. 2000). HPLC analyses (Table 2) demonstrated that there is an increase of carotenoids well known to be involved in photoprotection mechanism such as violaxanthin, anteraxanthin and zeaxanthin (Niyogi, K. K. et al. 1997) but also of a particular secondary carotenoid, the cantaxanthin, which is not detected in all algal species and is also possibly involved in protection from oxidative stress (Bidigare, R. R. et al. 1993).

The alteration in pigments composition is normally an indication of the modulation of photosynthetic complexes. The evaluation of proteins involved in photochemistry showed a relative reduction of PSII with respect to PSI, evidenced by both Western Blotting and ECS analyses (figures 2 and 3). As reported by [11] one likely explanation for PSII sensitivity is the rapid turnover of D1 in illuminated cells which requires efficient protein biosynthesis. In fact even under dim illuminations cells need to repair

the damaged protein complexes (Nixon et al., 2010) and nitrogen deprivation could determine a reduction in PSII repair cycle efficiency.

This reduced repair could lead to a stronger electron flux on residual functioning PSII resulting in a faster saturation of reaction centers which could probably determine an increased production of Reactive Oxygen Species (ROS). The alteration of photosystems stoichiometry in favor of PSI could be also contribute to prevent the formation of ROS at PSII level (Collier, J. L. et al. 1994) mainly directing electron flux to PSI.

It is worth mentioning that although PSII is the major target, PSI is also affected by nitrogen deprivation and its abundance was decreased by two fold.

Also the Cyt b_6f amount in N- cells decreased, confirming that nitrogen availability affects *Nannochloropsis* photosynthetic apparatus at many levels.

Thus presented data indicated that nitrogen deprivation mostly affects PSII while lowering the overall content of all the membrane complexes present in the thylakoid membranes.

4.5.3. *Nannochloropsis* exploits cyclic electron flow under nitrogen deprivation

Because PSII is the major target of nitrogen deprivation, *Nannochloropsis* starved cells cannot aliment linear electron flow at a sufficient rate to compete with the rates of the cytochrome b_6f complex and PSI catalyzed processes. This suggests that other electron flow modes may be responsible for the maintaining of the growth capacity observed in nitrogen limited cultures. Among possible candidates, cyclic electron flow could play a relevant role because of its capacity to generate ATP for housekeeping purposes and its only requires for cytochrome b_6f and PSI activity. In this work measurements of electron transport in the two nitrogen condition support this hypothesis, showing that

while LEF diminished, CEF was strongly increased in N- cells (figure 7). This additional response to N deprivation confirmed the ability of *Nannochloropsis* to face off to environmental changes as reported for other algae by [11] which responded to the reduction of PSII proteins, as in this case, with a decrease of the amount of LEF from PSII to PSI improving other types of electron flow such as CEF around PSI. This increase of CEF in N- cells could play a double role: it could maintain a sufficient photosynthesis rate for supporting the metabolism and it could be involved in some protection mechanisms against the possible damages induced by toxic oxygen radicals that may occur when the electron flux in PSII is higher than in PSI (Collier, J. L. et al. 1994). Other alternative electron flows were stable in both conditions (figure 7), confirming CEF as a major strategy in *Nannochloropsis* to guarantee a good electron transport through the photosynthesis chain.

The calculations about the incidence of LEF, CEF and others in this work were done comparing the same cells number of the two nitrogen conditions. However, taking into account that in N- there is a reduced amount of chlorophyll and photosystems as well, it has to be noted that the components of the photosynthesis chain still present are enough efficient to support photochemistry reactions although this response has to be considered as one of the possible adaptive mechanisms determining the ability of *Nannochloropsis* to survive even under nitrogen deprivation since this mechanism of survival could be accompanied by other acclimative responses.

In addition to that, previous work demonstrated how *Nannochloropsis* cells under nutrients deprivation were still able to increase the biomass (Sforza, E. et al. 2011). In particular in this condition there was an increase of cells weight instead of cells number probably due to lipids accumulation, confirming that in nutrients limitation like N

deprivation *Nannochloropsis* is able to maintain a residual ability to exploit photosynthesis.

Concluding, in this work we provided a *portrait* of *Nannochloropsis* in which under nitrogen deprivation the decrease of photosynthetic apparatus components amount is a strategy to ensure a good efficiency for the residual proteins.

4.6. REFERENCE LIST

Bailleul,B., Cardol,P., Breyton,C., and Finazzi,G. (2010) Electrochromism: a useful probe to study algal photosynthesis. Photosynth.Res. **106**:179-189.

Berges,J.A., Charlebois,D.O., Mauzerall,D.C., and Falkowski,P.G. (1996) Differential Effects of Nitrogen Limitation on Photosynthetic Efficiency of Photosystems I and II in Microalgae. Plant Physiol **110**:689-696.

Bidigare,R.R., Ondrusek,M.E., Kennicutt,M.C., Iturriaga,R., Harvey,H.R., Hoham,R.W., and Macko,S.A. (1993) Evidence for A Photoprotective Function for Secondary Carotenoids of Snow Algae. Journal of Phycology **29**:427-434.

Boussiba,S., Vonshak,A., Cohen,Z., Avissar,Y., and Richmond,A. (1987) Lipid and Biomass Production by the Halotolerant Microalga Nannochloropsis-Salina. Biomass **12**:37-47.

Brown,J.S. (1987) Functional Organization of Chlorophyll a and Carotenoids in the Alga, Nannochloropsis salina. Plant Physiol **83**:434-437.

Buchel,C. and Garab,G. (1995) Electrochromic Absorbency Changes in the Chlorophyll-C-Containing Alga Pleurochloris-Meiringensis (Xanthophyceae). Photosynthesis Research **43**:49-56.

Cardol,P., Alric,J., Girard-Bascou,J., Franck,F., Wollman,F.A., and Finazzi,G. (2009) Impaired respiration discloses the physiological significance of state transitions in Chlamydomonas. Proc.Natl.Acad.Sci.U.S.A **106**:15979-15984.

Cardol,P., Forti,G., and Finazzi,G. (2011) Regulation of electron transport in microalgae. Biochim.Biophys.Acta **1807**:912-918.

Chisti,Y. (2008) Biodiesel from microalgae beats bioethanol. Trends Biotechnol. **26**:126-131.

Chisti,Y. and Yan,J.Y. (2011) Energy from algae: Current status and future trends Algal biofuels - A status report. Applied Energy **88**:3277-3279.

Collier,J.L., Herbert,S.K., Fork,D.C., and Grossman,A.R. (1994) Changes in the Cyanobacterial Photosynthetic Apparatus During Acclimation to Macronutrient Deprivation. Photosynthesis Research **42**:173-183.

Croce,R., Canino,g., Ros,F., and Bassi,R. (2002) Chromophore organization in the higher-plant photosystem II antenna protein CP26. Biochemistry **41**:7334-7343.

Crowley,P.B., Diaz-Quintana,A., Molina-Heredia,F.P., Nieto,P., Sutter,M., Haehnel,W., De la Rosa,M.A., and Ubbink,M. (2002) The interactions of cyanobacterial cytochrome c6 and cytochrome f, characterized by NMR. J.Biol.Chem. **277**:48685-48689.

Demmig-Adams,B. and Adams,W.W. (2000) Harvesting sunlight safely. Nature **403**:371-374.

Demmig-Adams,B., Adams,W.W., Barker,D.H., Logan,B.A., Bowling D.R., and Verhoeven,A.S. (1996) Using chlorophyll fluorescence to assess the fraction of absorbed light allocated to thermal dissipation of excess excitation. Physiologia Plantarum **98**:253-264.

Eltgroth,M.L., Watwood,R.L., and Wolfe,G.V. (2005) Production and cellular localization of neutral long-chain lipids in the haptophyte algae Isochrysis galbana and Emiliania huxleyi. Journal of Phycology **41**:1000-1009.

Farber,A. and Jahns,P. (1998) The xanthophyll cycle of higher plants: influence of antenna size and membrane organization. Biochim.Biophys.Acta **1363**:47-58.

Gouveia,L. and Oliveira,A.C. (2009) Microalgae as a raw material for biofuels production. J.Ind.Microbiol.Biotechnol. **36**:269-274.

Greene,R.M., Geider,R.J., and Falkowski,P.G. (1991) Effect of Iron Limitation on Photosynthesis in A Marine Diatom. Limnology and Oceanography **36**:1772-1782.

Grossman,A. (2000) Acclimation of Chlamydomonas reinhardtii to its nutrient environment. Protist. **151**:201-224.

Grunewald,K., Hagen,C., and Braune,W. (1997) Secondary carotenoid accumulation in flagellates of the green alga Haematococcus lacustris. European Journal of Phycology **32**:387-392.

Guillard,R.R.L. and Ryther,J.H. (1962) Studies of marine planktonic diatoms. I. Cyclotella nana Hustedt and Detonula confervagea Cleve. Can.J.Microbiol. **8**:229-239.

Hodgson,P.A., Henderson,R.J., Sargent,J.R., and Leftley,J.W. (1991) Patterns of Variation in the Lipid Class and Fatty-Acid Composition of Nannochloropsis-Oculata (Eustigmatophyceae) During Batch Culture .1. the Growth-Cycle. Journal of Applied Phycology **3**:169-181.

Inda,L.A., Erdner,D.L., Peleato,M.L., and Anderson,D.M. (1999) Cytochrome c(6) isolated from the marine diatom Thalassiosira weissflogii. Phytochemistry **51**:1-4.

Joliot,P. and Delosme,R. (1974) Flash-induced 519 nm absorption change in green algae. Biochim.Biophys.Acta **357**:267-284.

Kolber,Z., Zehr,J., and Falkowski,P. (1988) Effects of Growth Irradiance and Nitrogen Limitation on Photosynthetic Energy Conversion in Photosystem II. Plant Physiol **88**:923-929.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680-685.

Lepetit,B., Volke,D., Szabo,M., Hoffmann,R., Garab,G., Wilhelm,C., and Goss,R. (2007) Spectroscopic and molecular characterization of the oligomeric antenna of the diatom Phaeodactylum tricornutum. Biochemistry **46**:9813-9822.

Li,Z., Wakao,S., Fischer,B.B., and Niyogi,K.K. (2009) Sensing and responding to excess light. Annu.Rev.Plant Biol. **60**:239-260.

Lubian,L.M., Montero,O., Moreno-Garrido,I., Huertas,I.E., Sobrino,C., Gonzalez-del Valle,M., and Pares,G. (2000) Nannochloropsis (Eustigmatophyceae) as source of commercially valuable pigments. Journal of Applied Phycology **12**:249-255.

Malcata,F.X. (2011) Microalgae and biofuels: a promising partnership? Trends Biotechnol. **29**:542-549.

Maxwell,K. and Johnson,G.N. (2000) Chlorophyll fluorescence - a practical guide. J.Exp.Bot. **51**:659-668.

Miller,R., Wu,G., Deshpande,R.R., Vieler,A., Gartner,K., Li,X., Moellering,E.R., Zauner,S., Cornish,A.J., Liu,B., Bullard,B., Sears,B.B., Kuo,M.H., Hegg,E.L., Shachar-Hill,Y., Shiu,S.H., and Benning,C. (2010) Changes in transcript abundance in Chlamydomonas reinhardtii following nitrogen deprivation predict diversion of metabolism. Plant Physiol **154**:1737-1752.

Moran,R. and Porath,D. (1980) Chlorophyll determination in intact tissues using n,n-dimethylformamide. Plant Physiol **65**:478-479.

Murata,N., Takahashi,S., Nishiyama,Y., and Allakhverdiev,S.I. (2007) Photoinhibition of photosystem II under environmental stress. Biochim.Biophys.Acta **1767**:414-421.

Niyogi,K.K., Björkman,O., and Grossman,A.R. (1997) The roles of specific xanthophylls in photoprotection. Proc.Natl.Acad.Sci.USA **94**:14162-14167.

Peltier,G. and Schmidt,G.W. (1991) Chlororespiration: an adaptation to nitrogen deficiency in Chlamydomonas reinhardtii. Proc.Natl.Acad.Sci.U.S.A **88**:4791-4795.

Peltier,G., Tolleter,D., Billon,E., and Cournac,L. (2010) Auxiliary electron transport pathways in chloroplasts of microalgae. Photosynth.Res. **106**:19-31.

Philipps,G., Happe,T., and Hemschemeier,A. (2011) Nitrogen deprivation results in photosynthetic hydrogen production in Chlamydomonas reinhardtii. Planta.

Porra,R.J., Thompson,W.A., and Kriedemann,P.E. (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim.Biophys.Acta **975**:384-394.

Richardson,B., Orcutt,D.M., Schwertner,H.A., Martinez,C.L., and Wickline,H.E. (1969) Effects of nitrogen limitation on the growth and composition of unicellular algae in continuous culture. Appl.Microbiol. **18**:245-250.

Rodolfi,L., Chini,Z.G., Bassi,N., Padovani,G., Biondi,N., Bonini,G., and Tredici,M.R. (2009) Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol.Bioeng. **102**:100-112.

Sforza,E., Bertucco,A., Morosinotto,T., and Giacometti,G.M. (2011) Photobioreactors for microalgal growth and oil production with Nannochloropsis salina: from lab-scale experiments to large-scale design. Chemical Engineering Research & Design **in press**.

Simionato,D., Sforza,E., Corteggiani,C.E., Bertucco,A., Giacometti,G.M., and Morosinotto,T. (2011) Acclimation of Nannochloropsis gaditana to different illumination regimes: Effects on lipids accumulation. Bioresour.Technol. **102**:6026-6032.

Sukenik,A., Beardall,J., Kromkamp,J.C., Kopecky,J., Masojidek,J., van Bergeijk,S., Gabai,S., Shaham,E., and Yamshon,A. (2009) Photosynthetic performance of outdoor Nannochloropsis mass cultures under a wide range of environmental conditions. Aquatic Microbial Ecology **56**:297-308.

Wellburn,A.R. (1994) The Spectral Determination of Chlorophyll-A and Chlorophhyll-B, As Well As Total Carotenoids, Using Various Solvents with Spectrophotometers of Different Resolution. Journal of Plant Physiology **144**:307-313.

Zhekisheva,M., Boussiba,S., Khozin-Goldberg,I., Zarka,A., and Cohen,Z. (2002) Accumulation of oleic acid in Haematococcus pluvialis (Chlorophyceae) under nitrogen starvation or high light is correlated with that of astaxanthin esters. Journal of Phycology **38**:325-331.

CHAPTER 5

EFFECTS OF NITROGEN AVAILABILITY ON *Chlamydomonas reinhardtii* PHOTOSYNTHETIC APPARATUS

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5.1. INTRODUCTION

Nitrogen (N) is a major constituent of the cell, being part of both proteins and nucleic acids and its availability has a huge impact on living organisms. This is particularly true for photosynthetic organisms which rely on other sources of energy as carbon and often are limited for their growth from the environmental availability of nutrients such as nitrogen. Carbon and nitrogen metabolism are closely linked, since the assimilation of nitrate to amino acids and other nitrogenous compounds requires reducing equivalents and carbon skeletons determining a high consumption of energy. On the other side, the rate limiting step of photosynthesis, i.e. carbon fixation (Young et al., 2012), needs nitrogenous compounds as proteins for the exploitation of electron transport and thus of the functionality of photosynthetic reactions. In this sense, nitrogen starvation determines a lower proteins content that could limit the flow of electrons through the photosynthetic apparatus affecting the entire photosynthetic efficiency (Hockin et al., 2012). Among photosynthetic organisms, algae have a particular interest because of their extreme diversity and their versatility in response to different conditions (as reviewed by Cardol et al, 2011) such as nutrients limitation.

Since nitrogen plays a fundamental role in algae growth, biomass productivity is often nitrogen concentration – dependent. In fact in nitrogen deprivation there is a reduced biomass production (Lubian et al., 1986, Chen et al., 2011, Hockin et al., 2012), while if nutrients are provided abundantly algae can give rise to "blooms". This is the case, for example, of humans/agricultural wastes rich in nitrates which can increase nutrients availability in coastal environments with a consequent explosion of algal proliferation (Sellner et al., 2003). Algae are also interesting organisms for biomass production because of their faster growth rate with respect to higher plants (Chisti, 2008, Gouveia and Oliveira, 2008). In this context nitrogen is also important because its deprivation

has been shown to induce accumulation of energy rich molecules such as lipids (Richardson et al., 1969, Guschina and Harwood, 2006, Rodolfi et al., 2008) or starch (Grossman, 2000, Siaut et al., 2011).

The availability of this macronutrient affects all aspects of algal physiology, including photosynthesis and determines different kind of response in algal cells. Concerning photosynthesis, nitrogen availability is known to affect pigments content inducing a reduction of chlorophyll content and an increase in carotenoids (Lubian et al., 1986, Grünewald et al., 1997, Zhekhishewa et al., 2002, Forjan et al., 2007). Nitrogen deprivation affects photosynthesis efficiency (Miller, 2010) and in diatoms and green algae it has been shown to affect in particular Photosystem II (Kolber et al., 1988, Berges et al., 1996). On the contrary, Photosystem I seemed to be more resistant to nitrogen deprivation, while being more sensitive to the absence of other nutrients such as iron (Greene et al., 1991, Berges et al., 1996). Nitrogen deprivation was also shown to inhibit Cytochrome b_6f complex (Bulté and Wollman, 1992).

In this work the response of *C. reinhardtii* photosynthetic apparatus to nitrogen deficiency was globally investigated, analyzing systematically how it responds to this stress condition. Results presented showed that *Chlamydomonas* responds to nitrogen deprivation by re-organizing the entire photosynthesis chain, reducing the content in PSII, PSI and Cyt b_6f complex and altering the stoichiometry of all this components. In these conditions a decrease in the linear electron flow efficiency was shown to be partially compensated by an increase in the activity of cyclic electron flow around PSI.

5.2. MATERIALS AND METHODS

5.2.1. Culture conditions

Chlamydomonas reinhardtii strain WT8b+ was grown in batch cultures under a continuous dim light of 100 μE $m^{-2}s^{-1}$. The cultures were grown in a TAP medium (Gorman and Levine, 1965) where the nitrogen source was guaranteed by adding NH_4Cl which resulted in a total 7 mM nitrogen concentration. Maintenance and propagation of cultures were performed using the same medium added with 10 g/l of Plant Agar (Duchefa Biochemie). Growth experiments were performed in Erlenmeyer flasks with magnetic agitation, starting from a pre-culture grown at 100 μE $m^{-2}s^{-1}$ at exponential phase which was diluted to an Optical Density (OD) value at 750 nm equal to 0.1, corresponding to ~$0.5*10^6$ cells/ml, final volume 150 ml. Algal growth was maintained for 2 days (pre-inoculum) and then cells were centrifuged at 2500g (Allegra 250, Beckman) for 10 minutes at RT, re-suspended at OD = 0.1 in two different TAP media, one with the same fresh medium and another one where nitrogen was present only in micromolar traces (0.5 μM). The growth was then performed for other 2 days. Illumination was constantly provided at 100 μE $m^{-2}s^{-1}$, using daylight fluorescent lamps. Temperature was kept at 23 ± 1 °C in a growth chamber. Algal growth was measured spectrophotometrically by daily changes in OD at 750 nm (Lambda Bio 40 UV/VIS Spectrometer, Perkin Elmer) and cells number monitored with a Bürker Counting Chamber (HBG, Germany) under light microscope. All curves were repeated at least four times.

5.2.2. Pigments extraction and analyses

Chlorophyll and total carotenoids were extracted from *Chlamydomonas* centrifuged cells at RT with 80% acetone for 5 minutes in dark conditions as in (Porra et al., 1989).

After 10 minutes of centrifugation at 10000g, the supernatant pigments concentration was determined spectrophotometrically using specific extinction coefficients (Porra et al., 1989, Wellburn, 1994).

HPLC analyses were carried out on the same samples using a Beckman System Gold instrument equipped with an UV-VIS rilevator and a 18-C column (Zorbax Ods, 25cm x 4.6 mm). 200 µl of sample were loaded and runs were performed as in (Farber and Jahns, 1998) using acetonitrile 86.7%, methanol 9.6% and Tris/HCl pH 7.8 3.6% as solvent A and methanol 80%, hexane 20% as solvent B. The peaks of each sample were identified through the retention time and absorption spectrum (Jeffrey et al., 1997) and each chromatogram was normalized on the Chl *a* peak to compare carotenoids. For the picomolar quantification of carotenoids correction factors as in (Farber and Jahns, 1998) were used. Each analysis was performed in triplicate.

5.2.3. Western Blot analyses on proteins from isolated thylakoids

Isolation of thylakoid membranes was performed according to (Lepetit et al., 2007) with some modifications. Cells from selected phase (before and after the re-suspension in the two nitrogen conditions) were harvested by centrifugation (Allegra 250, Beckman) at 4000 g for 5 minutes and then re-suspended in a variable volume of 10 mM MES pH 6.5, 2 mM KCl, 5 mM EDTA and 1 M Sorbitol (Isolation medium A, IMA). All following steps were performed at 4°C under dim light. Cells were centrifuged at 4000 g for 5 minutes and concentrated to an OD_{750} value equal to 50 with IMA added with 0.5% milk powder and protease inhibitors (1mM PMSF, 1mM DNP-ε-amino-*n*-caproic acid and 1mM benzamidine). The cells were disrupted with French Press at pressure of 0.9 kBar for one cycle. Unbroken cells were separated from the broken ones by a centrifugation step at 2500g for 10 minutes, then re-suspended in an equal volume of

IMA with 0.5% of milk powder and inhibitors as previously described, processed through French Press at 0.9 kBar and centrifuged as before twice. Broken cells of the three previous steps were mixed and subjected to a new French Press cycle at 0.9 kBar and centrifuged at 15000g for 20 min (5417R, Eppendorf). The pellet was re-suspended in a variable volume of 10 mM MES pH 6.5, 2 mM KCl, and 5 mM EDTA (Isolation medium B, IMB) and centrifuged at 15000g for 20 min. This step was repeated twice. Finally, the pellet composed of thylakoids was re-suspended in a small amount of 50 mM Hepes/KOH pH 7.5, 5 mM $MgCl_2$ and 50% glycerol (T3 buffer) and immediately freezed in liquid nitrogen and stored at -80°C. Total Chls were extracted with 80% acetone and the concentration was determined spectrophotometrically using specific extinction coefficients (Porra et al., 1989) and the fitting as in (Croce et al., 2002). SDS-PAGE analysis was performed with a Tris–Glycine buffer system as in (Laemmli, 1970) with 12 % acrylamide. For the evaluation of D1, PsaA and Cyt b_6f content, solubilized thylakoids were loaded using 4 μg Chl tot for D1 and PsaA and 2 μg Chl tot for Cyt b_6f . The solubilization buffer was 30% glycerol, 125 mM Tris pH 6.8, 0.1 M DTT, 9% SDS (SB3X). Western Blot analyses were performed using nitrocellulose (Pall Corporation) afterward incubated with specific antibodies.

5.2.4. In vivo monitoring of photosistem II quantum yeld

Chlorophyll fluorescence was determined in vivo using Dual PAM 100 from WALZ. The parameters Fv/Fm and NPQ were calculated respectively as (Fm-Fo)/Fm and (Fm-Fm')/Fm' (Demmig-Adams et al., 1996, Maxwell and Johnson, 2000) after 20 minutes of dark adaptation. During NPQ measurements a saturating light at 6000 μE $m^{-2}s^{-1}$ and an actinic light of 830 μE $m^{-2}s^{-1}$ were used.

5.2.5. Spectroscopic analyses on photosynthetic components: Electrochromic Shift

Following the same method described in the previous chapter, the in vivo spectrum of the Electrochromic Shift in *Chlamydomonas reinhardtii* in the 480-550 nm range upon continuous illumination of 1000 μE $m^{-2}s^{-1}$ was produced. All the measurements concerning absorption spectroscopy were performerd for cells first harvested, then re-suspended in a medium lacking of acetate and incubated 30 minutes at RT under agitation. Then PSI and PSII content was estimated spectroscopically from changes in the amplitude of the fast phase of the ECS signal at 520 nm, where the positive peak was located, and 546 nm, as basal line, upon excitation with a saturating laser flash (520 nm, 5 ns duration). PSII contribution was calculated from the decrease in the signal amplitude upon addition of DCMU (20 μM) and hydroxylamine (1 mM) that irreversibly block PSII charge separation once the sample has been pre illuminated. Conversely, PSI was estimated as the fraction of the signal that was insensitive to these inhibitors. Cytochromes b_6f complex and P700 were estimated by evaluating the maximum absorption change in samples incubated with DBMIB (10 μM) which inhibits cytochrome b_6f complex. Cyt b_6f amount was evaluated by subtracting absorption at 554 nm from a baseline drawn at 546 nm. P700 was measured at 705 nm. Measurements of linear, cyclic and other alternative electron flows were also deduced by measuring PSI turnover first in untreated cells and then incubated with DCMU (20 μM) to block linear flow and DCMU (20 μM) and DBMIB (10 μM) to block both linear and cyclic flow. CEF was then evaluated as the residual rate of $P700^+$ reduction that was observed in the presence of DCMU, but abolished by DBMIB addition. All spectroscopic measurements were performed using a JTS-10 (BioLogic, France).

5.3. RESULTS

5.3.1. Effects of nitrogen excess or limitation on *Chlamydomonas* growth

Chlamydomonas reinhardtii strain WT8b+ was grown in batch cultures under a continuous dim light of 100 μE $m^{-2}s^{-1}$. The cultures were grown in a TAP medium where the nitrogen source was guaranteed by adding NH_4Cl which resulted in a total 7 mM nitrogen concentration. Cells were grown for 2 days in a N rich medium and before reaching the stationary phase they were harvested and split in two cultures, one with the same fresh medium and another one where nitrogen was present only in micromolar traces. Growth kinetics, reported in figure 1, showed that *Chlamydomonas* growth rate is strongly affected by N deprivation. In fact, two days after the re-suspension in the two different N conditions, N- culture presented less than the 50% of cells respect with N+ culture.

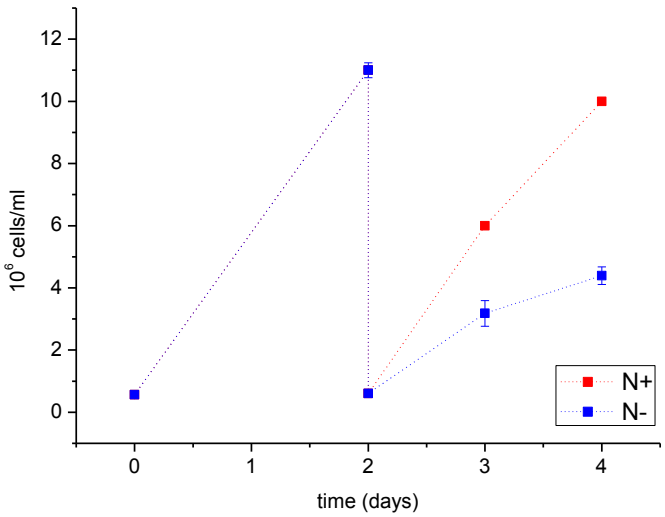


Figure 1. Growth of *Chlamydomonas reinhardtii* cultures with Nitrogen excess (red) or Nitrogen limitation (blue). The medium was N rich until day 2 and then cells were centrifuged and re-suspended in an N replete or N deprived medium for other 2 days.

5.3.2. Photosynthetic acclimation to different N conditions: modulation of pigments and protein content

In order to investigate the effects on *Chlamydomonas* photosynthetic apparatus of this depletion, first the pigment composition was analyzed. As shown in table 1, the pigments amount is strictly correlated with N availability. In fact N+ cells showed a total Chl content ~ 4 times higher respect with N- cells. As also shown in Table 1, Chl/car ratio strongly decreased in N- cells, suggesting a relative increase in carotenoids content. HPLC analyses (table 2) showed that lutein is the most abundant carotenoid in *Chlamydomonas* cells in both nitrogen conditions even if its amount is strongly increased in N- condition. Data in table 2 also showed the accumulation of carotenoids involved in photoprotection mechanisms such as violaxanthin, anteraxanthin and zeaxanthin (Niyogy et al., 1997, Demmig-Adams and Adams, 1992, Demmig-Adams and Adams, 2002).

Table 1. Pigment composition of N excess or N limited cells. Chl tot content per 10^6 cells and Chl/car (μg/μg) ratio in cultures before (pre-inoculum) and 2 days after the resuspension in the two N conditions are reported.

Nitrogen condition	μg chl tot / 10^6 cells	Chl/car
Pre-inoculum	7.198 ± 0.73	3.699 ± 0.168
N+	7.447 ± 0.89	3.424 ± 0.383
N-	1.97 ± 0.50	1.804 ± 0.047

Table 2. Carotenoids composition of *Chlamydomonas* cells before and after the re-suspension in N excess or N deprived conditions. The number of carotenoid moles over 100 Chl *a* moles in the two N conditions from HPLC analyses are reported.

Nitrogen condition	Neo+Loro	Viola	Antera	Lute	Zea	β- car
Pre-inoculum	17.5±0.7	5.3±0.2	0.4 ± 0.1	25.9 ± 0.1	0	6.6±0.15
N+	23.6±0.12	7.8±0.5	0.6 ± 0.2	29.1 ± 0.37	0	4.7±0.1
N-	5.6±0.3	15.8±0.14	3.9 ± 0.5	55.5 ± 0.2	3.9 ± 0.2	11.8±0.2

Normally, the reorganization of both Chls and carotenoids content is the result of the modulation of photosynthetic chain components. In order to verify if N deprivation affected specifically some of these complexes we evaluated the amount of D1 of PSII, PsaA of PSI and cyt b_6f complex in *Chlamydomonas* cultures adapted to N excess and N deprivation using specific antibodies. Western blotting in figure 2 showed a clear decrease of D1 content when cells are grown in N deprived medium (figure 2A). On the contrary, PsaA in N- condition showed an increased amount respect with N excess culture (figure 2B). It is worth pointing out that samples were loaded using equivalent Chl amounts to ensure a significant signal level. Western Blot analyses on other proteins involved in the photochemistry such as Cyt f (figure 2C) showed that this component content was maintained, always with respect to the Chl content.

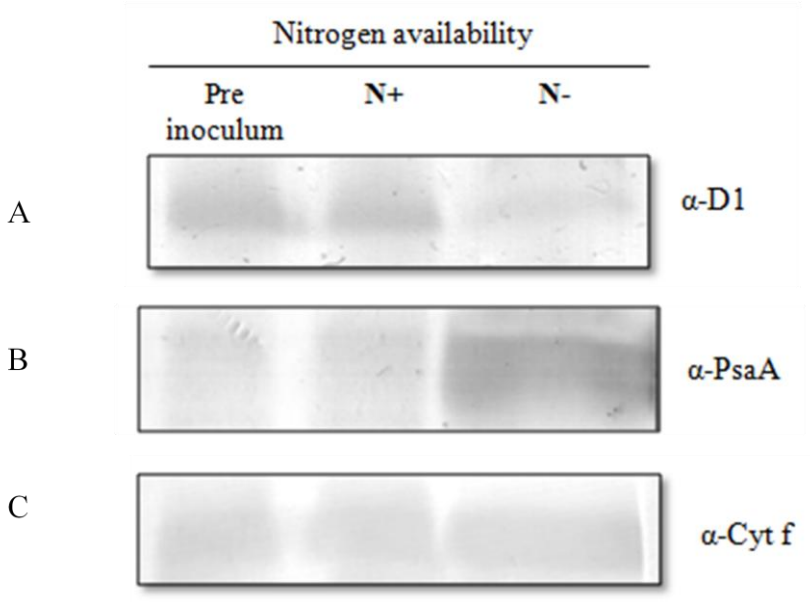


Figure 2. Photochemical efficiency of N excess and N deprived cells. Evaluation of A) D1, B) PsaA and C) Cyt f, determined using specific antibodies. Thylakoids were loaded containing 4 µg of Chl *a* for D1 and PsaA and 2 µg for Cyt f (thylakoids).

5.3.3. Photosynthetic apparatus composition in different N conditions

In order to investigate in more detail *Chlamydomonas* photosynthetic apparatus composition in response to N deprivation, in both N conditions it was analyzed using Electrochromic Shift (ECS). As reported in the previous chapter, ECS kinetics exploit the ability of carotenoids to modify their absorption spectrum during the exposure to continuous high light (Bailleul et al., 2010a). The most abundant carotenoid in *Chlamydomonas* is lutein although other carotenoids are present in the cells: loroxanthin, neoxanthin, violaxanthin, anteraxanthin, zeaxanthin and β-carotene (Polle et al., 2005) (Table 2). The absorption spectrum of *Chlamydomonas* determined with ECS analysis (Bailleul et al., 2010a) showed a positive peak at 520 nm. By focusing on the positive maximum at 520 nm it was possible to quantify PSII/PSI ratio in the two different N conditions as explained in previous chapter (Joliot and Delosme, 1974, Bailleul et al., 2010a). Using saturating laser flashes, in fact, ECS signal derives from both PSII and PSI. Their individual contribution to ECS signal is distinguishable using DCMU and HA which specifically affects PSII. In fact in presence of these inhibitors the only contribution to the ECS comes from PSI, allowing also for the calculation of PSII contribution by difference (Bailleul et al., 2010a). When this measurement was repeated for N+ and N- cells (figures 3A and 3B), ECS signal showed an alteration of photosystems relative composition. As shown in figure 3B there is a clear decrease in PSII abundance with respect to PSI in N- cells. Their different contribution can be precisely quantified, showing that PSII/PSI ratio was 1.01 ± 0.11 for N+ cells and 0.39 ± 0.06 for N- cells.

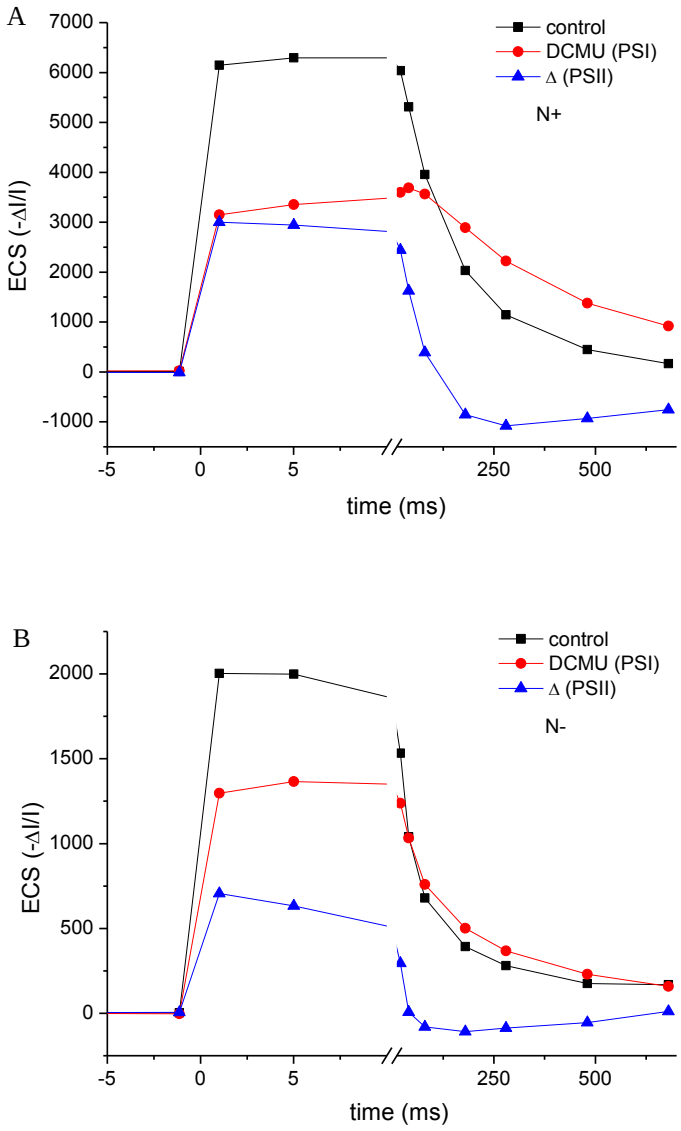
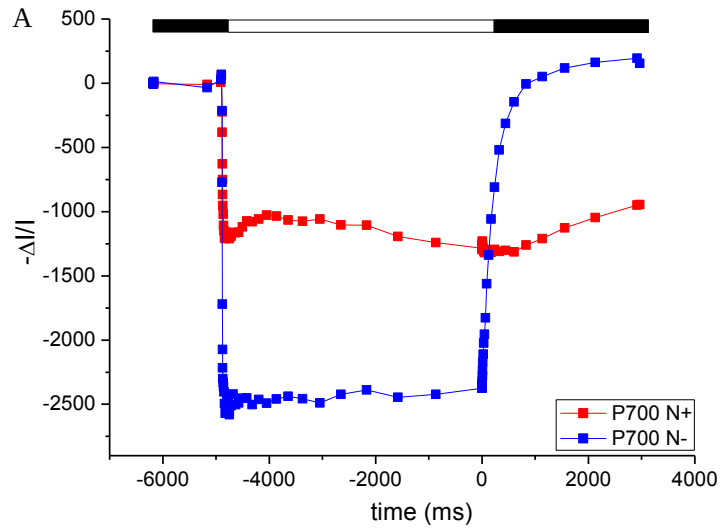
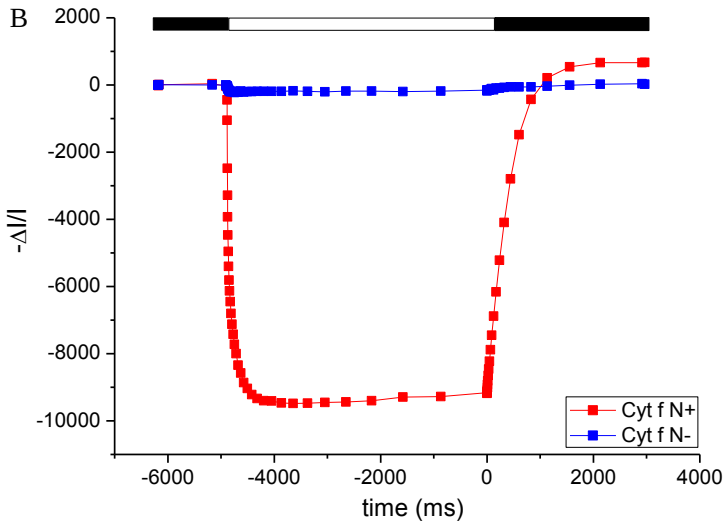


Figure 3. ECS kinetics measured in intact cells of *Clamydomonas* during a saturating laser pulse excitation. A) ECS kinetics at 520 nm in *Chlamydomonas* N+ cells for evaluating PSII/PSI ratio B) ECS kinetics at 520 nm in *Chlamydomonas* N- cells for evaluating PSII/PSI ratio. A) and B) Black squares = control kinetic, red circles = kinetic after the addition of DCMU and HA, blue triangles = kinetic after the addition of DCMU, HA and DBMIB.

Absorption spectroscopy was also exploited for the absolute quantification of some components of the photosynthesis chain like P700 and Cyt b_6f complex. P700 could be detected from a differential absorption signal around 700 nm (figure 4A), using a light source specifically exciting this photosystem (Bailleul et al., 2010a). These measurements showed that PSI amount per cells decreased under nitrogen deprivation

(figure 4C). Another fundamental component of photosynthetic apparatus is the cyt b_6f complex which together with PSII and PSI guarantees the electron transfer and generates a proton electrochemical potential gradient across thylakoid membranes (Yan et al., 2006). Absorption difference kinetics on N+ and N- cells in presence of DCMU, HA and DBMIB, an artificial quinone able to stabilize the charge separation (Itoh et al., 1996) and to inhibit Cyt b_6f complex activity (Yan et al., 2006) showed in this work a strong decrease of Cyt f under nitrogen deprivation (figures 4B and 4C).





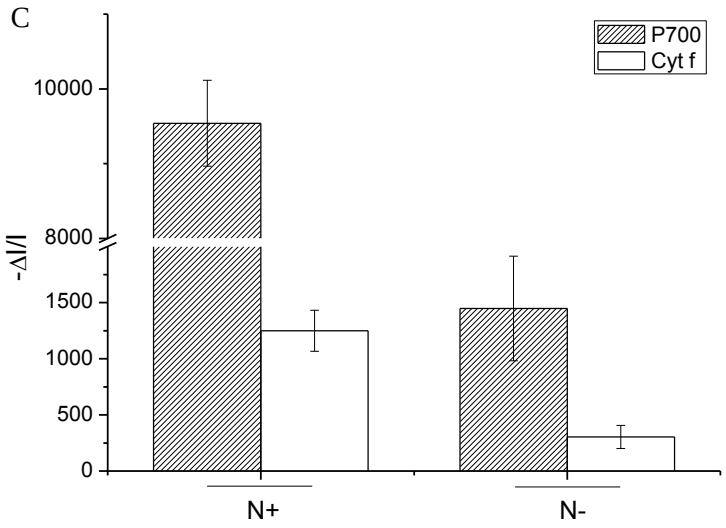


Figure 4. Differential absorption spectroscopy measured in intact cells of *Chlamydomonas* during a saturating laser pulse excitation. Data referred to the same cells number. Example for the detection of absolute content of A) P700 at 705 nm and B) Cyt f at 554 nm. A) and B) red squares = N excess cells, Blue squares = N deprived cells. Black barrel = light switched off during the measurement, empty barrel = light switched on during the measurement. C) P700 and Cyt f average values for all replicates.

5.3.4. Electron transport efficiency in different N conditions

Results presented above showed that nitrogen deprivation in *Chlamydomonas* cells affected most of the complexes in the electron transport chain. After assessing the composition of the photosynthetic apparatus was necessary to analyze how nitrogen deprivation affects also its activity. For this reason the fluorescence parameter Fv/Fm which allows the evaluation of PSII quantum yield (Maxwell and Johnson, 2000, Murata et al., 2007) was monitored in N+ and N- cells. Two days after the re-suspension in N excess or N deprived medium, cells showed how nitrogen deprivation affects also PSII efficiency (figure 5), although the reduction was not drastic.

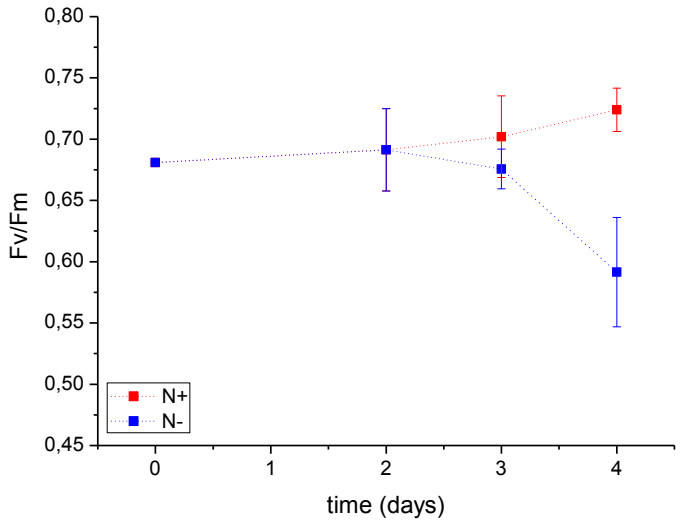


Figure 5. Photosynthetic parameters in *Chlamydomonas* cells acclimated to different N conditions. During growth curves reported in figure 1 Photosystem II efficiency was also monitored by determining the fluorescence parameter Fv/Fm. Here data for N excess and N limited cultures.

Electron transport efficiency was also evaluated; normally the Linear Electron Flow (LEF) is the major pathway for electron transport with PSII and PSI working in series (Joliot and Johnson, 2011). Alternative electron transport pathways have been described in plants and algae such as Cyclic Electron Flow (CEF) around PSI (see reviewed by Peltier et al., 2010), hydrogen production (Philipps et al., 2011) and chloro-respiration (Peltier and Schmidt, 1991). *Chlamydomonas* cells under N limitation showed a reduced total electron flow, as evidenced in figure 6. Using inhibitors like DCMU and DBMIB which affect specifically PSII and Cyt b_6f complex it was also possible to quantify the portion of LEF, CEF and other alternative electron flows. Results showed in figure 6 evidenced that in N+ cells the electron flow is almost completely due to LEF. On the contrary, in N- cells the overall decreased total electron flow was accompanied by a strong increase of cyclic transport. Absorption spectroscopy measurements using DBMIB also evidenced the presence of other alternative electron flows which

represented a very small part of total electron flow and were stable in both conditions of N availability.

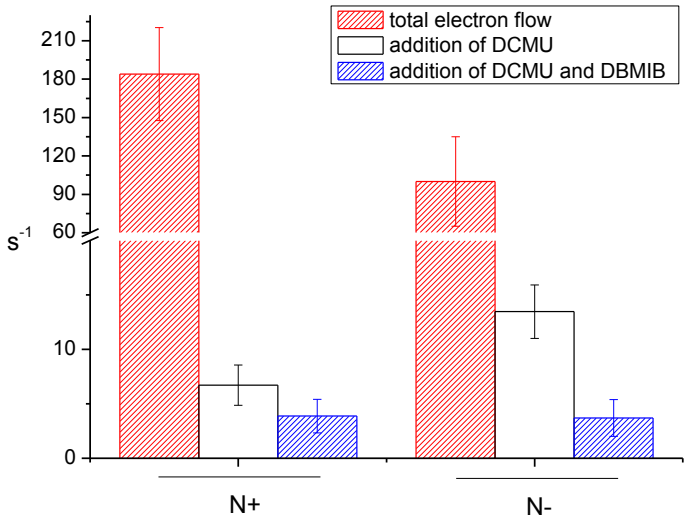


Figure 6. Spectroscopic quantification of the total and alternative electrons flow under N excess and N deprivation conditions in *Chlamydomonas* cells. This parameter has to be referred at the same cells number. The red column represents the total electron flow. The white column shows the contribution of cyclic and other alternative electron flows after the addition of DCMU. Red column minus white column represents the linear electron flow. The blue column refers to other alternative electron flows after the addition of DCMU and DBMIB which block respectively linear and cyclic electron flow. White column minus blue column represents the cyclic electron flow.

5.3.5. Activation of Non Photochemical Quenching (NPQ)

Photosynthetic organisms are continuosly exposed oxidative stress due to excess illumination. Several protection mechanisms evolved to increase their ability to survive in a variable environment. Among them Non Photochemical Quenching (NPQ) is particularly interesting for its ability to dissipate excess energy as heat when light exceeds the capacity for electron transport in order to prevent ROS production (Eberhard et al., 2008). Recently it was demonstrated that during nitrogen deprivation, *Chlamydomonas* accumulates large amount of a protein, PSBS, well known to be strictly involved in the activation of NPQ in *Arabidopsis thaliana* (Miller et al., 2010). Thus, the NPQ process was monitored in dark adapted *Chlamydomonas* cells showing

that in both N+ and N- cells this mechanism was not strongly activated, reaching values below 1 (figure 7). While this was expected for N+ cells since they were not exposed to strong light this also means that N deprivation while affecting electron transport efficiency, as evidenced by the different fluorescence kinetics, it does not induce NPQ.

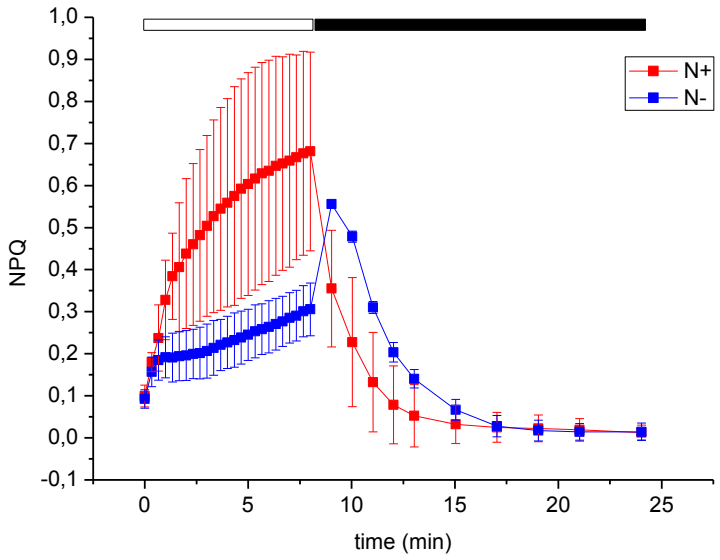


Figure 7. Non-photochemical quenching (NPQ) in *Chlamydomonas* N+ and N- cells. Data for N excess cells (red) and N deprived cells (blue) are reported. Measurements are done at least 4 times. 830 µE $m^{-2}s^{-1}$ actinic light was switched off after 8 min. Empty barrel = light switched on during the measurement, black barrel = light switched off during the measurement.

5.4. DISCUSSION

5.4.1. *Chlamydomonas* responds to nitrogen availability trough the re-organization of the entire photosynthetic apparatus

In this work *Chlamydomonas* cultures grown in a N rich and N depleted medium were compared. Cultures showed that nitrogen deprivation causes a growth limitation with a decreased biomass production as suggested by other studies (Grossman, 2000, Miller et al., 2010). In this work, the principal aim was to systematically investigate the response

of *Chlamydomonas* photosynthetic apparatus to nitrogen deprivation in order to improve the understanding of algae adaptation mechanisms to environmental changes.

Since the first analyses, it appears clear that nitrogen deprivation showed a huge impact on photosynthetic apparatus. In fact pigment content was largely affected by this stress with chlorophyll amount per cell in N- cells strongly decreased (Table 1), as expected since their biosynthesis requires nitrogen. An increase in relative carotenoid content was also observed as normally happens in response to oxidative stress in photosynthetic organisms (Grünewald et al., 1997, Li et al., 2009). This increase in carotenoids is mainly due to lutein which is known to play a role in protection from oxidative stress (Polle et al., 2001, Guedes et al., 2011). Other carotenoids whose content is increased are violaxanthin, antheraxanthin and zeaxanthin which are well known to play a role in photoprotection participating to the xanthophylls cycle (Niyogi et al., 1997, Demmig-Adams and Adams, 2000).

Neoxanthin and loroxanthin content is instead strongly diminished probably as a result of a reduction of antenna systems, where this carotenoids are specifically bound. This is in agreement with previous work in which Chlamydomonas mutants lacking loroxanthin and neoxanthin showed a reduced PSII light-harvesting Chl antenna size (Polle et al., 2001). Under nitrogen deprivation cells showed also a decreased amount in β-carotene and this is in accord with previous works which confirmed the ability of modulating β-carotene content for algae belonging to Chlorophyceae as *Chlamydomonas* as a common response to nitrogen availability (Abd-El-Baki et al., 2004, Guedes et al., 2011). It should be mentioned that a similar response in pigment content was also found in *Chlamydomonas* acclimated to high light conditions (Bonente et al., 2012). Comparing this aspect with that of *Nannochloropsis* (Table 2, Chapter 4)

appears clear how the increase of carotenoids involved in photoprotection mechanism seems to be a common response to nitrogen deprivation in algae.

Since pigments are strictly linked to the protein complexes of the photosynthesis chain, an alteration of pigments is often reflected in the protein specific pattern of the photosynthetic apparatus. This hypothesis was confirmed by Western Blot reported in figure 2 and ECS analyses (Figures 3A and 3B) which showed the alteration of the balance between the two photosystems in response to N availability in *Chlamydomonas* cells. PSII was confirmed as the major target under N deprivation as previously suggested (Berges et al., 1996) and one of the likely explanation is that PSII subunit D1 undergoes a rapid turnover which is affected by nitrogen deprivation (Kim et al., 1993, Berges et al., 1996, Nixon et al., 2010). This is confirmed by chlorophyll fluorescence analyses in this work which showed a decrease in PSII quantum yield (figure 5).

Despite PSII is the major target of nitrogen deprivation it has to be underlined that in this condition other components of the electron transport chain are affected. PSI is known to be seriously affected by iron deficiency (Greene et al., 1991) but we demonstrated that despite increasing its content relatively to PSII, nitrogen deprivation strongly influence PSI absolute amount in the cells (figures 4A and 4C). In addition, Cyt b_6f complex was strongly affected by nitrogen availability as well, registering a huge decrease of absolute amount in *Chlamydomonas* N- cells (figures 4B and 4C). This result is also in agreement with a previous work (Bulté and Wollman, 1992) in which nitrogen deprivation stimulated the gametogenesis in *Chlamydomonas* cells with a following loss of Cyt b_6f.

Thus, data presented until now assessed the influence on nitrogen availability at many levels in the photosynthesis chain, determining a general re-organization of the entire photosynthetic apparatus.

5.4.2. *Chlamydomonas* improves cyclic electron flow to overcome nitrogen deprivation

In addition of photosynthetic complexes stoichiometry also photosynthetic efficiency was altered. PSII efficiency decreased (figure 5) and the linear electron flow from PSII to PSI diminished as well (figure 6). However, in N- cells the cyclic electron flow strongly increased. Thus the exploitation of alternative electron flows instead of the classical LEF could be interpreted with a double role. In fact it could be a strategy to maximize the residual photosynthetic efficiency but also CEF could be useful for the promotion of ATP synthesis through the Cyt b_6f complex (Alric, 2010). Comparing this results with *Nannochloropsis* response in the previous chapter, the re-organization of the entire photosynthetic apparatus and the improvement of alternative electron flows instead of LEF, seem to be a common response to nitrogen deficiency in algae even not strictly correlated from an evolutive point of view.

5.4.3. Activation of Non Photochemical Quenching

Photosynthetic organisms are well known to be prone to oxidative stress due to excess illumination and several protection mechanisms evolved to increase their ability to survive in a variable environment. Among them Non Photochemical Quenching (NPQ) is particularly able to allow plants and algae to overcome such situation of stress (Eberhard et al., 2008). This process requires for its activation a strong proton gradient (Joliot and Johnson, 2011) and a protein act as a "facilitator". Interesting different proteins has been shown to play this role in plants and algae, respectively PSBS and LHCSR (Li et al., 2002, Peers et al., 2009, Bailleul et al., 2010b). Previous studies showed that while *Chlamydomonas reinhardtii* NPQ depends from LHCSR, a gene encoding for PSBS is present in its genome, although the protein is never found

accumulated in the thylakoids (Bonente et al., 2008, Peers et al., 2009). Recently it has been shown that nitrogen depletion induce PSBS gene expression (Miller et al., 2010) suggesting NPQ could be activated in these peculiar conditions in *Chlamydomonas*. However, in this work was demonstrated that NPQ in N+ and N- cells was not strongly activated (figure 7). During the first illuminated part of the measurement N+ cells showed higher values respect with N- cells while when light was switched off the NPQ relaxed immediately. On the contrary N- cells showed a peculiar arise of NPQ immediately after the light switch off, which in any case slowly relaxed for the ATP-sinthase activity. An explanation for this behavior might be attributed to the chloro-respiration activity which has been suggested to increase in nitrogen depletion (Peltier et al., 1991). In fact this process could contribute to an increase of a trans-thylakoidal pH gradient in the first seconds of the dark phase of the measurement (Peltier et Cournac, 2002) with a consequent increase in NPQ. In this sense, it could be possible that the proton gradient in N- cells during the light phase was not sufficient to increase the NPQ while the contribution of chloro-respiration during the first minutes of the dark phase was fundamental for the enhanced activation of NPQ in N- cells. On the contrary, because of the supposed lower presence of chloro-respiration under nitrogen excess and the activity of ATP synthase which requires protons, in N+ cells in the dark phase there was not the same increment of the trans-thylakoidal pH gradient. Consequently, N+ cells did not showed the same behavior of N deprived cells.

5.5. REFERENCE LIST

Abd El-Baky H. H., El Baz F. K. and El-Baroty G. S. (2004). Production of antioxidant by the green alga Dunaliella salina. International Journal of Agricultural and Biology.

Alric, J. (2010). Cyclic electron flow around Photosystem I in unicellular green algae. Photosynthesis Research.

Bailleul B., Cardol P., Breyton C. and Finazzi G. (2010a). Electrochromism: a useful probe to study algal photosynthesis. Photosynthesis Research.

Bailleul B., Rogato A., de Martino A. , Coesel S., Cardol P., Bowler C., Falciatore A. and Finazzi G. (2010b). An atypical member of the light-harvesting complex stress related protein family modulates diatom responses to light. PNAS.

Berges J. A., Charlebois D. O., Mauzerall D. C. and Falkowski P. G. (1996). Differential effects of nitrogen limitation on photosynthetic efficiency of Photosystems I and II in microalgae. Plant Physiology.

Bonente G., Passarini F., Cazzaniga S., Mancone C., Buia M. C., Tripodi M., Bassi R. and Caffarri S. (2008). The occurrence of the psbS gene product in Chlamydomonas reinhardtii and in other photosynthetic organisms and its correlation with energy quenching. Photochemistry and Photobiology.

Bonente G., Pippa S., Castellano S., Bassi R. and Ballottari M. (2012). Acclimation of Chlamydomonas reinhardtii To Different Growth Irradiances. Journal of Biological Chemistry.

Bulté L. and Wollman A. F. (1992). Evidence for a selective destabilization of an integral membrane protein, the Cytochrome b_6f complex, during gametogenesis in Chlamydomonas reinhardtii. European Journal of Biochemistry.

Cardol P., Forti G. and Finazzi G. (2011). Regulation of electron transport in microalgae. Biochimica et Biophysica Acta.

Cardol P., Alric J., Girard-Bascou J., Franck F., Wollman F. A. and Finazzi G. (2009). Impaired respiration discloses the physiological significance of state transitions in Chlamydomonas. PNAS.

Chen M., Tang H., Maa H., Holland T. C., Simon Ng K. Y. and Salley S. O. (2011). Effect of nutrients on growth and lipid accumulation in the green algae Dunaliella tertiolecta. Bioresource Technology.

Chisti Y. (2008). Biodiesel from microalgae beats bioethanol. Trends in Biotechnology.

Collier J. L., Herbert S. K., Fork D. C. and Grossman A. R. (1994). Changes in the cyanobacterial photosynthetic apparatus during acclimation to macronutrient deprivation. Photosynthesis Research.

Croce R., Canino G., Ros F. and Bassi R. (2002). Chromophore organization in the higher-plant photosystem II antenna protein CP26. Biochemistry.

Demming-Adams B. and Adams W. W. (1992). Photoprotection and other responses of plants to high light stress. Annual Review of Plant Physiology and Plant Molecular Biology.

Demmig-Adams B., Adams W. W., Barker D. H., Logan B. A., Bowling D. R. and Verhoeven A. S. (1996). Using chlorophyll fluorescence to assess the fraction of absorbed light allocated to thermal dissipation of excess excitation. Physiologia Plantarum.

Demmig-Adams. B. and Demmig. W. W. (2002). Antioxidants in Photosynthesis and Human Nutrition. Science.

Eberhard S., Finazzi G. and Wollman A. F. (2008). The dynamics of photosynthesis. Annual Review of Genetics.

Farber A. and Jahns P. (1998). The xanthophyll cycle of higher plants: influence of antenna size and membrane organization. Biochimica et Biophysica Acta.

Forjan I., Garbayo I., Casal C. and Vílchez C. (2007). Enhancement of carotenoid production in Nannochloropsis by phosphate and sulphur limitation. Communicating Current Research and Educational Topics and Trends in Applied Microbiology.

Gorman D. S. and Levine R. P. (1965). Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of Chalmydomonas reinhardtii. PNAS.

Gouveia L. and Oliveira A. C. (2009). Microalgae as a raw material for biofuels production. Journal of Industrial Microbiology and Biotechnology.

Green B. R. and Geider R. J. and Falkowski P. G. (1991). Effect of iron limitation on photosynthesis in a marine diatom. Limnology Oceanography.

Grossman A. (2000) Acclimation of Chlamydomonas reinhardtii to its nutrient environment. Protist 151: 201-224

Grunewald K., Hagen C. and Braune W. (1997). Secondary carotenoid accumulation in flagellates of the green alga Haematococcus lacustris. European Journal of Phycology.

Guedes A. C., Amaro H. M. and Malcata F. X. (2011). Microalgae as sources of carotenoids. Marine Drugs.

Guschina I. A. and Hartwood J. L. (2006). Lipids and lipid metabolism in eukaryotic algae. Progress in Lipid Research.

Hockin N. L., Mock T., Mulholland F., Kopriva S. and Malin G. (2012). The Response of Diatom Central Carbon Metabolism to Nitrogen Starvation Is Different from That of Green Algae and Higher Plants. Plant Physiology.

Itoh S., Iwaki M., Tomo T. and Satoh K. (1996). Dibromothymoquinone (DBMIB) Replaces the Function of QA at 77 K in the Isolated Photosystem II Reaction Center (Dl-D2-Cytochrome 6559) Complex: Difference Spectrum of the P680+(DBMIB) State. Plant Cell Physiology.

Jeffrey S. W., Mantoura R. F. C. and Wright S. W. (1997). Data for the identification of 47 key phytoplankton pigments. In: Phytoplankton pigments in oceanography. UNESCO Publishing, Paris.

Joliot P. and Delosme R. (1974). Flash-induced 519 nm absorption change in green algae. Biochimica et Biophysica Acta.

Joliot P. and Johnson G. N. (2011). Regulation of cyclic and linear electron flow in higher plants. PNAS.

Kim J. H., Nemson J. A. and Melis A. (1993). Photosystem II reaction center damage and repair in Dunaliella salina (Green Alga). Analysis under physiological and irradiance stress conditions. Plant Physiology.

Kolber Z. , Zehr J. and Falkowski P. (1988). Effects of Growth Irradiance and Nitrogen
Limitation on Photosynthetic Energy Conversion in Photosystem II. Plant Physiology.

Laemmli U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature.

Lepetit B., Volke D., Szabo M., Hoffmann R., Garab G., Wilhelm C. and Goss R. (2007). Spectroscopic and molecular characterization of the oligomeric antenna of the diatom Phaeodactylum tricornutum. Biochemistry.

Li Z., Wakao S., Fischer B. B. and Niyogi K. K. (2009). Sensing and responding to excess light. Annual Review of Plant Biology.

Lubiàn L. M., Establier R. and Ruiz E. (1986). La luz y el nitrato en el crescimiento y contenido de pigmentos en cultivos de Nannochloropsis gaditana Lubiàn. Investigacion Pesquera.

Maxwell K. and Johnson G. N. (2000). Chlorophyll fluorescence - a practical guide. Journal of Experimental Botany.

Merchant S. S., Allen M. D., Kropat J., Moseley J. L., Long J. C., Tottey S., Terauchi A. M. (2006). Between a rock and a hard place: Trace element nutrition in Chlamydomonas. Biochimica et Biophysica Acta.

Miller R., Wu G., Deshpande R. R., Vieler A., Gartner K., Li X., Moellering E. R., Zauner S., Cornish A. J., Liu B., Bullard B., Sears B. B., Kuo M. H., Hegg E. L., Shachar-Hill Y., Shiu S. H. and Benning C. (2010). Changes in transcript abundance in Chlamydomonas reinhardtii following nitrogen deprivation predict diversion of metabolism. Plant Physiology.

Murata N., Takahashi S., Nishiyama Y. and Allakhverdiev S.I. (2007). Photoinhibition of photosystem II under environmental stress. Biochim Biophys Acta.

Nixon P. J., Michoux F., Yu J., Boehm M. and Komenda J. (2010). Recent advances in understanding the assembly and repair of Photosystem II. Annals of Botany.

Niyogi K. K., Bjorkman O. and Grossman A. R. (1997). Chlamydomonas Xanthophyll Cycle mutants identified by video imaging of chlorophyll fluorescence quenching. The Plant Cell.

Peers G., Truong T. B., Ostendorf E. Busch A., Elrad D., Grossman A. R., Hippler M., Niyogi K. K. (2009). An ancient light-harvesting protein is critical for the regulation of algal photosynthesis. Nature.

Peltier G. and Cournac L. (2002). Chlororespiration. Annual Review of Plant Biology.

Peltier G., Tolleter D., Billon E. and Cournac L. (2010). Auxiliary electron transport pathways in chloroplasts of microalgae. Photosynthesis Research.

Philipps G., Happe T. and Hemschemeier A. (2011) Nitrogen deprivation results in photosynthetic hydrogen production in Chlamydomonas reinhardtii. Planta

Polle J. E. W., Niyogi K. K. and Melis A. (2001). Absence of the pigments lutein, violaxanthin and neoxanthin affects the functional chlorophyll antenna size of Photosystem II but not that of Photosystem I in the green alga Chlamydomonas reinhardtii. Proceedings of the 2001 DOE Hydrogen Program Review.

Porra R. J., Thompson W. A. and Kriedemann P. E. (1989). Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochimica et Biophysica Acta.

Richardson B., Orcutit D. M., Schwertner H. A., Martinez C. L. and Wickline A. H. E. (1969).Effects of Nitrogen Limitation on the Growth and Composition of Unicellular Algae in Continuous Culture. Applied Microbiology.

Rodolfi L., Chini Z. G., Bassi N., Padovani G., Biondi N., Bonini G. and Tredici M. R. (2008). Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnology and Bioengineering.

Sellner K. G., Doucette G. J. and Kirkpatrick G. J. (2003). Harmful algal blooms: causes, impacts and detection. Journal of Industrial Microbiology and Biotechnology.

Siaut M., Cuiné S., Cagnon C., Fessler B., Nguyen M., Carrier P., Beyly A., Beisson F., Triantaphylidès C., Li-Beisson Y. and Peltier G. (2011). Oil accumulation in the model green alga Chlamydomonas reinhardtii: characterization, variability between common laboratory strains and relationship with starch riserve. BMC Biotechnology.

Wellburn A. R. (1994). The Spectral Determination of Chlorophyll-a and Chlorophhyll-b, As Well As Total Carotenoids, Using Various Solvents with Spectrophotometers of Different Resolution. Journal of Plant Physiology.

Yan J., Kurisu G. and Cramer W. A. (2006). Intraprotein transfer of the quinone analogue inhibitor 2,5-dibromo-3-methyl-6-isopropyl-pbenzoquinone in the cytochrome b_6f complex. PNAS.

Young J. N., Rickaby R. E. M., Kapralov M. V. and Filatov D. A. (2012). Adaptive signals in algal Rubisco reveal a history of ancient atmospheric carbon dioxide. Philosophycal Transaction of the Royal Society.

Zhekhishewa M., Boussiba S., Khozin-Goldberg I., Zarka A. and Cohen Z. (2002). Accumulation of oleic acid in Haematococcus pluvialis (chlorophyceae) under nitrogen starvation or high light is correlated with that of astaxanthin esters. Journal of Phycology.

APPENDIX 1

OPTIMIZATION OF PHOTOSYNTHETIC EFFICIENCY FOR ALGAE GROWING IN A PHOTOBIOREACTOR USING ALTERNATION OF LIGHT AND DARK CYCLES

Eleonora Sforza[1], Diana Simionato[2], Giorgio Mario Giacometti[2], Alberto Bertucco[1] and Tomas Morosinotto[2] contributed to this work

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ABSTRACT

Biofuels from algae are highly interesting as renewable energy source to at least partially replace fossil fuels. However, in order to develop large-scale cultivation systems with sufficient productivity research efforts are still needed to optimize growth parameters. Light has a major relevance for algae and, if too low, it limits algae growth while if in excess it drives to oxidative stress. Light use efficiency in algae large-scale cultivation systems must thus be optimized to achieve a sufficient productivity to make the process economically sustainable. In this work the influence of light intensity on growth and lipids productivity of *Nannochloropsis salina* was investigated using a flat-bed photobioreactor designed to minimize cells self-shading. The influence of different light intensities was investigated using both continuous illumination and alternation of light and dark cycles with different frequencies, which mimic illumination variations in a photobioreactor due to mixing. Results show that *Nannochloropsis* is able to use efficiently even under very intense light, provided that dark cycles are present to allow for re-oxidation of photosynthetic apparatus electron transporters. Instead, if alternation of light and dark is not optimal, algae experience radiation damages and photosynthetic productivity is strongly reduced. Presented results suggest that in an algae photobioreactor mixing optimization is seminal for algae to exploit light energy with high efficiency.

APPENDIX 2

IDENTIFICATION OF KEY RESIDUES FOR pH DEPENDENT ACTIVATION OF VIOLAXANTHIN DE-EPOXIDASE FROM *ARABIDOPSIS THALIANA*

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ABSTRACT

Plants are often exposed to saturating light conditions, which can lead to oxidative stress. The carotenoid zeaxanthin, synthesized from violaxanthin by Violaxanthin De-Epoxidase (VDE) plays a major role in the protection from excess illumination. VDE activation is triggered by a pH reduction in the thylakoids lumen occurring under saturating light.

In this work the mechanism of the VDE activation was investigated on a molecular level using multi conformer continuum electrostatic calculations, site directed mutagenesis and molecular dynamics.

The pKa values of residues of the inactive VDE were determined to identify target residues that could be implicated in the activation. Five such target residues were investigated closer by site directed mutagenesis, whereas variants in four residues (D98, D117, H168 and D206) caused a reduction in enzymatic activity indicating a role in the activation of VDE while D86 mutants did not show any alteration. Molecular dynamics showed that the VDE structure was coherent at pH 7 with a low and constant amount of water penetrating the hydrophobic barrel. Simulations carried out with the candidate residues locked into their protonated state showed instead an increased amount of water penetrating the barrel and the rupture of the H121-Y214 hydrogen bond, which is essential for VDE activation. Additionally, the analysis of the VDE sequence showed that the four putative activation residues are all conserved in plants but not in diatoms, explaining why VDE in these algae is already activated at higher pH. This suggests that VDE activation relies on a robust and redundant network.

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